Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Medicine
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Platelets, cholestatic liver disease and cystic fibrosis

Peter WITTERS

Doctoral thesis in Biomedical Sciences

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Dankwoord

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A teacher affects eternity; he can never tell where his influence stops (Henry Adams)

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List of Abbreviations

2-MeSADP: 2-Methyl-thio-ADP
AA: Arachidonic acid
ADP: Adenosine diphosphate
ALA: Alpha-linoleic acid
ALT Alanine transaminase
AST Aspartate transaminase
ATP: Adenosine triphosphate
AUC: Area under the curve
BDL: Bile duct ligation
BMI: Body mass index
cAMP: cyclic adenosine monophosphate
CF: Cystic fibrosis
CFF: Cystic Fibrosis Foundation
CFLD: Cystic fibrosis associated liver disease
CFPH: Cystic fibrosis related portal hypertension
CFTR: Cystic fibrosis transmembrane conductance regulator
CI: Confidence interval
DHA: Docosahexaenoic acid
DMSO: Di-methylsulphoxide
EFA: Essential fatty acid
EPA: Eicosapentaenoic acid
FA: Fatty acid
FEV1: Forced expiratory volume (one second)
FVC: Forced vital capacity
GGT: Gamma-glutamyltransferase
GSH: Reduced glutathione
HGF: Hepatocyte growth factor
IQR: Interquartile range
ISC: Incomplete septal cirrhosis
LA: Linoleic acid
LT: Leukotriene
MA: Mead acid
NASH: Non-alcoholic steatohepatitis
NCPH: Noncirrhotic portal hypertension
NRH: Nodular regenerative hyperplasia
PBC: Primary biliary cirrhosis
PFA-100: Platelet function analyser
PG: Prostaglandin
PGE1: Prostaglandin E1
PGI2: Prostacyclin
PKA: Protein kinase A
PPP: Platelet poor plasma
PRP: Platelet rich plasma
PSC Primary sclerosing cholangitis
PT: Prothrombin time
PUFA: Poly-unsaturated fatty acid
ROC: Receiver operating characteristic
TAA: Thio-acetamide
TX: Thromboxane
VASP: Vasodilator-stimulated phosphoprotein

Introduction

1. Liver disease & platelets

Adapted from: P. Witters, K. Freson, C. Verslype, K. Peerlinck, M. Hoylaerts, F. Nevens, C. Van Geet, and D. Cassiman, "Review article: blood platelet number and function in chronic liver disease and cirrhosis." Alimentary Pharmacology & Therapeutics, vol. 27, Jun. 2008, pp. 1017-1029.

Haemostasis and hepatology are closely related. It is well-known that patients with cirrhosis show a marked decrease in liver synthesis of coagulation factors, which leads to a prolongation of the prothrombin time [1]. Less well studied, but equally important in the physiological process of clot formation, are the defects in the primary haemostasis in patients with liver disease. Blood platelets initiate the haemostatic process by interaction with the damaged vessel wall [2]. These form a haemostatic plug within seconds after injury. The secondary haemostasis (coagulation) starts simultaneously, but enrolls slower, and strengthens this plug by crosslinked-fibrin within minutes [2;3].

Cirrhosis is the final common end-point of many chronic liver diseases and thrombocytopenia is a well-known feature of cirrhosis. However, in addition to the quantitative changes in platelets, there are also qualitative defects. These can be assessed in specialized haemostatic laboratories. The most primitive but physiologically meaningful platelet function test is the *in vivo* bleeding time assessment [4]. More sensitive and reproducible tests have been developed, for instance assays that try to simulate the in vivo platelet function *in vitro*, under flow conditions, to study shear-induced platelet activation. These tests are technically demanding, but reflect aggregation in maximally physiological conditions [5]. Less physiological, but more widely used and currently the gold standard for platelet function assessment is the aggregometry [4;6]. This in vitro assay measures platelet aggregation in response to different sets of agonists (collagen, thrombin, adenosine diphosphate (ADP), etc.). Aggregometry is performed with platelet rich plasma (obtained by slight centrifugation of anticoagulated blood) where optical light turbidity is a measure of aggregation [7]. It can also be performed with whole blood by electrical impedance measurement [4]. Another sensitive assay is the quantification of membrane molecule expression by flow cytometry (FACS analysis). By assessing the presence of stimulation-dependent antigens (e.g. CD62P (P-selectin)) or platelet-leucocyte complexes, the degree of platelet activation can be estimated

[4;7]. Even more specialized, on a molecular level, the concentrations of second messengers (e.g. calcium, cyclic AMP) and the release of platelet granules containing pro-aggregatory molecules (serotonin, ATP, platelet factor 4, beta-thromboglobulin, etc.) can be assessed [4]. Finally, due to the technical difficulties with platelet function tests, several tests (as are thromboelastography and sonoclot analysis) have been developed, for everyday clinical use. These tests require little or no sample preparation and give instant bedside results to assist in clinical decision making [7]. In thromboelastography for example, blood clots in a backward and forward rotating sample cup around a suspended pin. Due to haemostasis the cup's motions become limited. These movements are mapped and different variables (depending on coagulation, fibrinolysis and platelet function) can be calculated [6;7].

Although spontaneous bleeding due to low platelet counts in cirrhosis is an infrequent event, low platelet counts become clinically relevant when performing liver biopsies, liver transplantation or giving myelosuppressive agents (antiviral treatment like interferon or cytostatics) [8]. Less well studied is the recent observation that platelets can contribute to the progression of liver disease (e.g. in viral hepatitis and cholestatic liver disease [9;10]). Finally platelet derived serotonin has convincingly proven to be involved in the initiation of liver regeneration [11].

The aim of this part of the introduction is to give an overview of the literature of blood platelets in cirrhosis and chronic liver disease.

1.1. Platelet Number in cirrhosis and chronic liver disease

Theoretically, lowered platelet counts can be the result of decreased platelet production, enhanced splenic sequestration or platelet consumption/use. Kinetic studies with radiolabeled platelets in cirrhosis and chronic liver disease indicate there is a decreased platelet survival [12-15]. Hereby platelet counts can be reduced [12] or normal [14] depending on the ability of the bone marrow to increase the platelet production. These findings are in accordance with the decreased [16;17], normal [18] or increased [19] percentage of reticulated platelets (young platelets) in cirrhosis. The main site of platelet consumption is in the spleen [12;13;15].

Overall, it is not yet clear what the exact mechanisms leading to thrombocytopenia in cirrhosis are. It is most likely a multifactorial process combining increased splenic platelet breakdown, splenic pooling and the inability of the bone marrow to increase platelet production adequately [20]. Most authors agree on a decrease in platelet count in relation to the *severity* of cirrhosis [21-23].

1.2. Platelet function

a) Platelet function in cirrhosis

The existence of a functional platelet defect (hypoaggregability) was described for the first time by Thomas et al. [24]. The paradigmal platelet function test, bleeding time, has been reported to be abnormal in 2,5- 42% of patients with cirrhosis [25-27]. This correlates only weakly with the platelet count, suggestive of an additional functional deficit. This bleeding time prolongation is closely related to the degree of liver failure [27].

Only few studies on platelets in flow conditions (the most physiologic test in vitro) have been performed [5;28-31]. These report conflicting results (decreased [5;31] and normal [28-30] platelet function) possibly due to differences in experimental setup as varying shear rates from 600 s^{-1} [31], 800 s^{-1} [5;30], 1600 s^{-1} [29] to 2600 s^{-1} [28] and differences in the adhesive surfaces that were perfused. Moreover, because these studies were performed in whole blood or reconstituted blood (with adjusted hematocrit and platelet count) other factors than platelet function could have played an important role. Indeed plasma factors and haemorheological factors dependent on the hematocrit are important in these experiments [29].

In humans and in animal models of chronic liver disease and cirrhosis there is a clear hypoaggregability, as demonstrated with in vitro aggregation tests (the gold standard) [5;28;32-41] and with flowcytometric analysis of stimulation dependent antigens (e.g. P-selectin) [37]. A decreased response to collagen [28;32-34 ;38;40;42], thrombin [33-36], arachidonic acid [5;28;34;37;40], ADP [5;28;32;37;39], U46619 (an ADP-mimetic) [5;28;42], epinephrine [5;28;37] and ristocetin [5;28;43] have been reported. These findings are also confirmed by thromboelastography [44;45]. Crossover experiments (using platelet aggregometry) with patient or control platelets in control or patient plasma hold both an intrinsic platelet defect and a circulating plasma factor responsible for this hypofunction [39;41].

The molecular mechanisms underlying this ***intrinsic platelet defect*** leading to hypoaggregability have been studied extensively. The most important defects are depicted in Figure 1: the normal platelet as compared to the cirrhotic platelet.

There is evidence supporting a reduced transmembrane signaling in cirrhotic platelets after stimulation with thrombin or collagen [33;34]. This leads to a decreased activation of phopholipase C, A2 and cyclooxygenase/thromboxane synthetase [33;34] resulting in

decreased thromboxane production [46]. There is also evidence for a decreased arachidonic acid availability for prostaglandin and thromboxane production [38;46]. Moreover, dietary supplementation with arachidonic acid increases the membrane levels of this fatty acid and improves (collagen-induced) platelet aggregation in cirrhosis, returning to pretreatment levels 4 weeks after withdrawal [38].

The IP3 production in response to thrombin stimulation is significantly lower in platelets from patients with cirrhosis [36]. There is a reduced cytosolic calcium increase, also due to a decreased release from intracellular stores [36]. As intracellular calcium is the final common pathway in platelet aggregation, this leads to platelet hypofunction. There is also a decreased cytosolic alkalinization (Na+/H+ antiporter activity) which normally facilitates calcium entry from the extracellular environment [36].

Furthermore, there is evidence for a storage pool defect in cirrhosis, decreasing the effect of granule release on the aggregation. There is decreased adenosine triphosphate (ATP) and serotonin (5HT) in the dense bodies and decreased PF4 (platelet factor 4), β-thromboglobulin (BTG) and P-selectin in the alpha granules [32;35;37;47]. These granules have a normal ultrastructure and normal relative volume [35]. Plasma levels of BTG and PF4 are reported to be elevated in relation to platelet count [35], although not all authors agree [47].

Finally, there is an upregulation of inhibitory pathways. Basal cyclic adenosine monophosphate and cyclic guanosine monophosphate, the two main inhibitory messengers, are upregulated in the cirrhotic platelet [36]. There was a normal upregulation upon stimulation indicating an increased stimulation of circulating factors in vivo like PGI_2 [48-50] and NO [51] for cAMP and cGMP respectively [36]. There is one study suggesting a locoregional difference in platelet aggregation, i.e. a difference in platelet aggregation between portal and, simultaneously drawn, systemic blood. Splanchnic vasodilatation and enhanced generation of PGI_2 shift the collagen (but not the ADP) aggregation curve significantly to the right in portal blood. There were no differences in coagulation and fibrinolytic parameters [49].

As possible underlying mechanism ‘platelet exhaustion’ was proposed, as platelets are faced with the portal hyperdynamic circulation. Platelets could be damaged during intravascular activation (with loss of granules), which could give rise to their subsequent hypo-function when tested in vitro [32]. This hypothesis is somewhat challenged by the normal microscopy of the platelets [35], the normal concentration of thrombin-antithrombin complexes, D-dimers

and F1+2 (fibrin(ogen) degradation products) values and the absence of stimulation-dependent antigens on the platelet membrane [37]. The increased urinary excretion of thromboxane A2 (TXA2) metabolites (2,3 dinor-thromboxane B2 (TXB2) and 11dehydro-TXB2) could be explained by intrasplenic destruction [37].

Currently defective transmembrane signaling (and secondarily a decrease of the intracellular messengers) is thought to be the most important factor in the platelet hypo-function [34;37].

Figure 1: Platelets in normal (a) and cirrhotic (b) condition

Figure a: In normal platelets, stimulation by collagen, thrombin, ADP and TXA/B2 leads to activation of the PLC (in green, left side of the figure). This results in the formation of DAG and IP3. DAG leads to activation of PLA2 and the production and release of TXA/B2. This reinforces the PLC activity and stimulates additional platelets. IP3 results in calcium release from the dense tubular system (stored through the SERCA pump). The increase of cytosolic calcium (also aided by the ADP P2X receptor and the H/Ca-Na-antiporter) moderates the platelet activation: the shape change (by actin); the release of stored pro-aggregatory molecules (ATP and 5HT from the dense bodies and BTG, PF4 and P selectin from the alfa granules) recruiting other platelets and the conformational change of the GPIIb/IIIa receptor (together with the phosphorylation by the PKC) allowing interactions with fibrin reinforcing the haemostatic plug. These mechanisms are under tight homeostatic control of the inhibitory pathways (in red, right side of the figure): cAMP (produced by AC: stimulated by adenosine, PGI2 and PGE2 and inhibited by ADP via the P2Y2 receptor) and cGMP (produced by the NO-stimulated GC).

Figure b: In cirrhosis, platelet activation is decreased (in green, left side of the figure). There is decreased PLC and PLA2 activity leading to decreased TXA/B2 production and decreased IP3 mediated cytosolic calcium increase. There is also less calcium transport by the H/Ca-Na-antiporter because of diminished intraplatelet acidification. The secondarily impaired release of the dense bodies and the alpha granules is less effective due to the storage pool defect: less ATP and 5HT; BTG, PF4 and P-selectin-levels in the graules respectively. The inhibitory pathways are also upregulated (in red, right side of the figure). This results in more cAMP- and cGMP-mediated inhibition. Finally, there are also negative plasma factors decreasing platelet activation as are the FDP and ApoE (See text for details).

Abbreviations on the figure: 5HT: serotonin, AC: adenylate cyclase, ApoE: Apolipoprotein E, ATP: adenosine-triphosphate, BTG: β-thromboglobulin, Ca: calcium, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, DAG: diacylglycerol, FDP: fibrin(ogen) degradation products, GPIIb/IIIa: glycoprotein IIb/IIIa, GTP: Guanosine triphosphate, H: hydrogen, IP3: inositol-triphosphate, Na: sodium, NO: nitric oxide, P2Y1, P2X1, P2TY2: ADP receptors, PC: phosphatidylcholine, PF4: platelet factor 4, PGE2: prostaglandin E2, PGI2: prostacyclin, PIP2: phosphatidylinositol-bisphosphate, PKC: protein kinase C, PLA2: phospholipase A2, PLC: phospholipase C, SERCA: sarco/endoplasmic reticulum Ca-ATPase, TXA2: thromboxane A2, TXB2: thomboxane B2.

Figure 1a

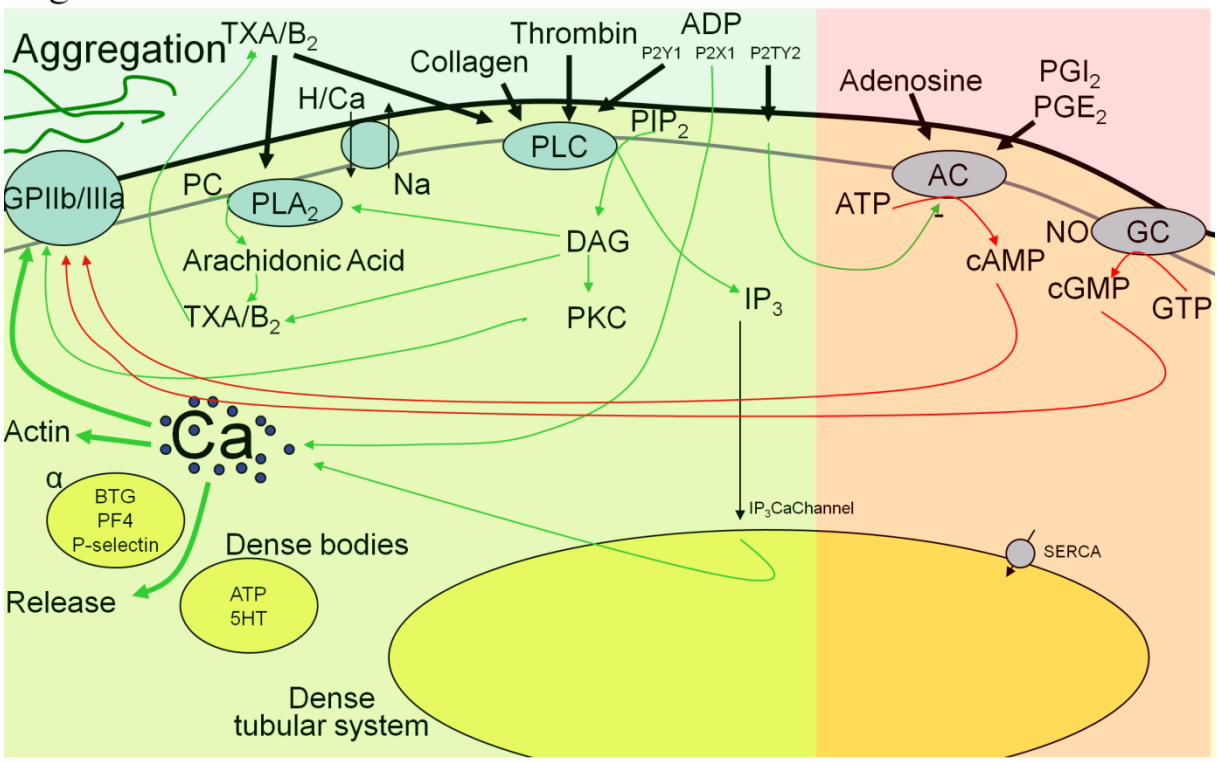
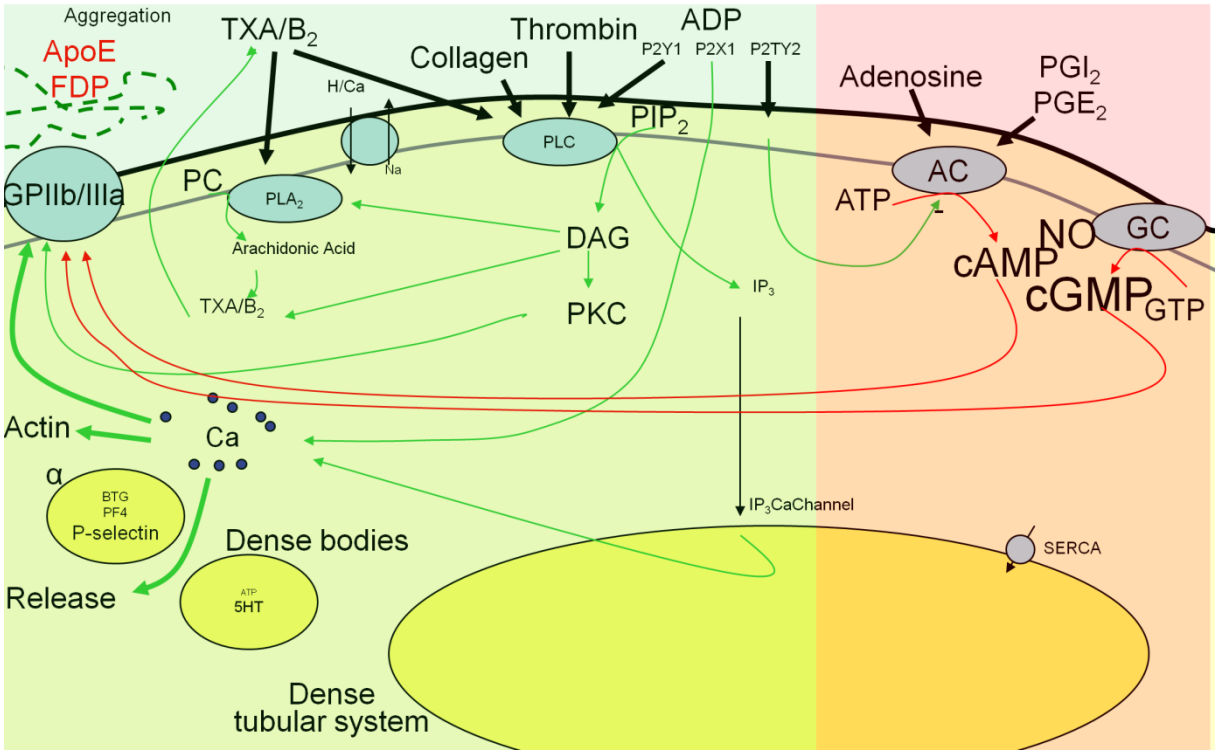


Figure 1b



Besides this intrinsic platelet defect various ***plasma factors*** also appear to play a role. Numerous negative factors are known.

HDL apolipoprotein E contents are increased in cirrhosis and this was correlated with the inhibition of platelet aggregation [52].

There are also *fibrin(ogen) degradation products*, that appear in excess in liver disease [53], that are known to adsorb to the platelet surface and interfere with platelet function [54]. However, their levels may not be well correlated with reduced platelet aggregation [55].

Bile salts can also exert a negative effect. In vitro aggregation studies show an inhibitory effect of bile salts on platelet aggregation and serotonin release induced by ADP or collagen [56]. Only chenodeoxycholic acid is an aggregation-inducing bile salt [56]. In vivo studies in rat models associated with elevated bile acids (bile duct ligation and cholic acid feeding) show a clear inhibitory effect on ADP-epinephrine induced aggregation [57].

Finally, as already stated, primary and secondary haemostasis are closely related. As defects in the *coagulation* are well-studied (for review see [2;58]) it is clear that they independently contribute to the haemostatic abnormalities in patients with liver disease. However, their impairment may also affect platelet function. For example, platelets play an important role in thrombin formation and thrombin is a platelet agonist [1]. It is interesting to note that the capacity of platelets to support thrombin generation in patients with cirrhosis is comparable to healthy controls provided that the platelet counts are adjusted to normal levels [59].

There are also positive factors that promote platelet activation e.g. *vWF* that is reported to be upregulated in cirrhotic patients [29]. Despite the reduced number of (more active) high molecular weight vWF multimers [29;60], the reduced collagen binding capacity and relative ristocetin activity (all indicative of loss of function), it is likely that the quantitative effect exceeds the qualitative effect [29]. Glycoprotein Ib (the vWF receptor) has been reported to be increased [53] or decreased [43] in cirrhosis. As already stated these plasma factors can contribute to the apparent discrepancy between the studies under flow conditions and the in vitro aggregation tests.

Unconjugated bilirubin is a strong inducer of platelet aggregation similar to ADP in isolated platelets. This could be completely inhibited by prostaglandin I_2 or E_2 [61]. However, the physiologic relevance of this observation has to be questioned since the effect was entirely

inhibited by the addition of 0.1% bovine serum albumin, probably due to the high affinity of albumin to unconjugated bilirubin [61].

Overall there is a clear platelet dysfunction. As for the intrinsic platelet defect several steps of platelet activation are shown to be impaired. Underlying mechanisms remain speculative. Furthermore the blood platelets from patients with liver disease encounter several agonists and antagonists. This could lead to hyper- and hypoaggregability, it is however not clear what the contribution of these isolated findings is in the complex entity of chronic liver disease and cirrhosis.

b) Platelet function in cholestatic liver disease

As with platelet number, also platelet function seems dependent on the degree of liver fibrosis [5;34;35]. Until now there has been little attention on the different liver diseases in relation to platelet abnormalities. Most reports are on 'cirrhotic platelets', however, in cholestatic liver disease there is some evidence that, contrary to other types of cirrhosis, the platelets demonstrate a hyperaggregability.

Patients with primary biliary cirrhosis (PBC) /primary sclerosing cholangitis (PSC) have a better survival after variceal bleeding [62] and demonstrate less blood loss during liver transplantation [63;64], although this can also be explained by a better synthetic liver function at the time of transplantation. Thrombosis of portal veins has also been detected in 40% of PBC livers at the time of transplantation [65]. There have been some platelet function studies in these patients that are indicative of hyperaggregability. This has been documented by the use of thromboelastography [66;67], by sonoclot analysis [68] (two newer and less well studied platelet tests that try to evaluate the complete haemostatic system) and PFA-100 analysis [67;68]. In the latter there is a lack of prolongation of closure time [67;68] as is normally seen in cirrhosis [28;67]. On a molecular level there is an increased expression of CD42b (an antigen of the gpIb/V/IX complex which binds to vWF) and of ligand-induced binding sites both in basal conditions and after stimulation with ADP or epinephrine [67]. This suggests that platelets are preactivated in PBC/PSC. This is consistent with the higher levels of thrombin-antithrombin complexes and increased levels of homocysteinemia and tissue factor as signs of endothelial activation [68]. Possible explanations for this hyperaggregability in PBC/PSC are the marked systemic inflammatory activity and the significantly higher levels of unconjugated bilirubin which might be pro-aggregable (cfr. supra) [67]. Futhermore, in a well-studied animal model of cholestasis (bile-duct ligated rats)

there is a rapid rise in the stimulated platelet cytosolic calcium, the final common pathway in platelet activation, to a greater amplitude compared to control animals [69]. This is due to a greater release from intracellular stores which store more calcium due to increased sacroplasmatic/endoplasmatic-reticulum calcium-ATPase activity [69].

Not all reports are unequivocal on this hyperaggregability as this could not be demonstrated by platelet aggregometry (the gold standard) in both patients [32] and animal models of PBC [42]. Moreover, there is an increased bleeding time with a trend to normalize after inhibition of nitric oxide production (a potent platelet antagonist) [42], a smaller amount of releasable ATP possibly due to loss of platelet granules during intravascular activation [32], and a lack of increase of CD62P after stimulation [67]; all indicative of decreased platelet function.

The underlying mechanism for altered platelet function in cholestasis is unclear. It could be due to an intrinsic change in platelet function (e.g. increased aggregation by altered calcium homeostasis [69]) or decreased aggregation due to a storage pool deficiency [32]) or due to the effect of a plasmatic factor (e.g. inhibition by bile acids [70] or stimulation by bilirubin [70]).

Also in cholestasis, a physiologic explanation for platelet hypoaggregability, ‘platelet exhaustion’ (intravascular activation with subsequent loss of granule secretion and hence hypoaggregability when tested in vitro) has been proposed [32;35;69] and refuted [37].

Very recently, the contribution of blood platelets to liver damage in cholestatic liver disease was studied in bile-duct ligated mice [10]. Platelet depletion or blockage of the P-selectin receptor of platelets led to decreased aggregation formation (within the liver), decreased platelet adhesion and less leucocyte accumulation resulting in improved liver transaminase levels [10].

2. Cystic fibrosis

Wehe dem Kind, das beim Kuß auf die Stirn salzig schmekt, er ist verhext und muss bald sterben.

Literature from Germany and Switzerland, around 1700

Cystic fibrosis (CF) is the most frequent lethal genetic disorder, within the Caucasian population, with an incidence of 1 to 2500-4000. CF is caused by a mutation in the *CFTR* gene (cystic fibrosis transmembrane conductance regulator). This encodes the cystic fibrosis transmembrane conductance regulator, a cAMP dependent chloride channel mainly responsible for chloride transport across apical membranes of epithelial surfaces [71]. More than 1700 mutations are known since its original description in 1989, with the Phe508del mutation being responsible for 70% of cases (http://www.genet.sickkids.on.ca/cftr/app).

At the time of its first recognition as a disease entity (1938), life expectancy was only months. In patients born today, a life expectancy of 50 years or more seems realistic. [72]. This improvement was only possible due to improved supportive care, as the search for correction therapy with restoring chloride channel and other CFTR functions is still ongoing.

CF is a multisystem disease. The most affected organ, and likewise the main cause of death, is the lung. Absence of the functional CFTR leads to increased viscosity of the exocrine secretions, leading to ciliary dysfunction, mucus impaction and infection in the lung [73]. There is chronic infection with *H. influenzae, S. aureus, P. Aeruginosa, B. cepacia, S. Maltophila, …* . End stage complications include severe haemoptysis, pneumothorax and ultimately respiratory failure [74]. Aggressive therapy and centralized patient care has led to better outcomes [72].

Besides the pulmonary disease, there are gastro-intestinal manifestations (meconium ileus, pancreatic insufficiency, malnutrition and liver disease); endocrine disorders (pancreatic dysfunction with CF related diabetes mellitus, osteoporosis) and infertility in males (congenital absence of vas deference) [72]. There are also more subtle metabolic disturbances such as changes in fatty acid composition of membranes of CF affected tissues and in the plasma [75] and alterations in the primary haemostasis (platelets) [76].

In all of these manifestations complete physiopathology has not yet been completely elucidated and a very active research community exists, trying to gain more insight and consequentially improve patients' outcome.

In the following parts of this introduction we will take a closer look on essential fatty acids, platelets and liver disease in cystic fibrosis.

2.1 Cystic fibrosis and essential fatty acids

Fish oil is a whale of a story, that not surprisingly gets bigger with every telling

Rogan JA, NEJM 1987;316:624-8

The pioneering observation of the very low incidence of some chronic inflammatory conditions in the native Inuit of Greenland (Eskimos consuming a traditional diet rich in fish oils) led to great interest in the field of essential fatty acids in health and disease [77].

In humans poly-unsaturated fatty acids (PUFAs) linoleic acid (LA, C18:2n-6) and alpha-linoleic acid (ALA, C18:3n-3) are 'essential' for normal growth and function. Their only source is dietary. Besides a role as energy source, they have several biological functions [77].

Poly-unsaturated fatty acids originating from the essential fatty acids (EFAs) by elongation and desaturation are incorporated in the cell membranes. There, they contribute to regulate physiochemical properties of membrane lipid matrix and influence a wide number of membrane-bound proteins [78]. Some PUFAs are precursors of biologically active, regulatory molecules, the eicosanoids. These compromise the prostaglandins (PG), prostacyclins, thromboxanes (TX) and leukotrienes (LT). They are potent second messengers and regulate various processes as inflammation, endothelial integrity, blood pressure, etc. [78]. More recently, EFAs are also acknowledged as modulators of gene expression via their action on peroxisome proliferator-activated receptors [78].

Alpha-linoleic (n-3) and linoleic acid (n-6) compete for the same enzymes for their conversion to eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) and arachidonic acid (AA) respectively. Eicosanoids deriving from n-3 fatty acids lead to less potent platelet aggregation and vasoconstriction (formation of TXA_3 instead of TXA_2), less active vasodilatation and platelet inhibition (PGI_3 instead of PGI_2) and less induction of inflammation and chemotaxis of and adhesion of leucocytes (LTB_3 instead of LTB_4)

compared to eicosanoids deriving from the n-6 fatty acid arachidonic acid (AA) [79]. So the balance between n-3 and n-6 determines the biological effect.

Beneficial effects of n-3 essential fatty acids have been shown in the secondary prevention of coronary heart disease, hypertension, type 2 diabetes, and, in some patients with renal disease, rheumatoid arthritis and inflammatory bowel disease [79;80]. Supplements are currently endorsed by the American Heart Association in patients with documented coronary heart disease.

In CF, in an effort to further improve care, more and more attention is paid to metabolic disturbances and hence EFA deficiency [78]. Abnormal fatty acid (FA) profile has been reported in children with cystic fibrosis since many years [81]. Renewed interest in this subject emerges from the ability to influence FA profiles by supplements, thereby adding to the dietary strategies to obtain an optimal nutritional state [78]. Moreover, disturbances in FA profiles have been implicated in the predisposition to lung disease [82]. Also, in a CF mouse model, supplementation of one of the FA (DHA) was beneficial [83]. Nevertheless, randomised controlled trials are needed to confirm or refute beneficial effects of EFA supplementation in CF patients [78].

The major EFA defect in CF patients seems to be an increased release of AA, the most important metabolic product of LA [84]. In serum as well as in nasal and rectal biopsy samples, a high ratio of AA to DHA is seen [75]. In addition, there is a decrease in plasma levels of LA (18:2 n-6) and DHA (C22:6 n-3) and a compensatory increase in levels of eicosatrienoic acid (20:3 n-9) (also known as mead acid (MA)) [78;85-87].

The aetiology of this abnormal fatty acid profile remains obscure. Initially this was thought to reflect fat malabsorption linked to the exocrine pancreatic insufficiency. However, it even occurs in well-nourished young cystic fibrosis patients [88] which makes this less probable. It has been suggested that there is a disturbance in essential fatty acid metabolism [89]. This disturbance is related to the CF-genotype and minor changes in the essential fatty acid patterns are even seen in CF-heterozygotes [75;90].

2.2 Cystic fibrosis and Platelets

Platelets are the main actors in primary haemostasis. Besides this vital function, the role of platelets in inflammation is increasingly appreciated. Actually, platelets can be seen as classic inflammatory cells. They produce and store enormous amounts of pro-inflammatory

mediators, they can cross-talk with and activate different cells and are in turn activated by multiple pro-inflammatory substances [91-93].

Recently, platelets have been suggested to contribute to the CF pulmonary inflammation and tissue destruction and it was hypothesized that platelet activation is an important event in CF lung disease [92;94].

It is not clear whether CF platelets demonstrate in vitro hyperaggregability or not. Hyperaggregability has been demonstrated by various authors [76;95;96], but could not be confirmed by others [46;93]. However, in physiological circumstances, platelet activation is counterbalanced by platelet inhibition in order to limit the spread of platelet activation and prevent a generalized prothrombotic state. This is among others controlled by the endothelium that produces eicosanoids (the inhibitory prostacyclin) which act locally on the platelets. In CF, abnormalities in the platelet inhibition by eicosanoids have been unequivocally demonstrated. PGE_1 is less capable of inhibiting ADP-induced platelet aggregation [97] and P-selectin expression (a marker for platelet activation) [76] in CF. A similar defect of PGE_1 on platelet volume has been shown [98].

Only very recently, expression of CFTR in human platelets has been demonstrated, which might be responsible for some of the changes in platelet function seen in CF, although the exact mechanisms still need to be elucidated [99]. Nevertheless, O'Sullivan et al. showed there is an additional effect of CF plasma on non-CF platelet function [76]. When whole blood from healthy controls is diluted with CF plasma, there is an increased expression of P-selectin on platelets and likewise, if CF blood is diluted with control plasma, there is a decrease in P-selectin expression [76]. This shows that the altered platelet function in CF is not only due to the absence/reduction of CFTR function in platelets, but also to a hitherto unidentified plasma-borne factor.

CF is characterized by an abnormal EFA state, especially decreased DHA levels [75;78]. Correcting this deficiency might ameliorate the course of the disease and several trials in patients looking at the effect of DHA supplements on lung function have been undertaken (be it with variable results) [78]. The effect of DHA deficiency on platelet function remains unstudied.

Interestingly, essential fatty acids (mainly omega-3 fatty acids) are known to modulate platelet activation. In vitro, DHA interferes with TXA_2 formation [100;101], is capable of

inhibiting the TX receptor [102] and could attenuate collagen-induced aggregation [100;101]. However, in randomized controlled trials in healthy controls an effect on platelet pro-aggregatory function could not be demonstrated [103]. The effect of eicosanoid-induced platelet inhibition however, remains to be studied.

2.3 Cystic fibrosis and liver disease

If the liver is stiff, the prognosis is bad.

Hippocrates, 460-370 B.C.

In CF, pulmonary problems are the main cause of morbity and mortality. However, CF associated liver disease (CFLD) is an upcoming health issue. Emerging during the second decade of life, it leads to significant morbidity and decreased quality of life in a selected population of young CF patients [104;105]. Moreover, it is the second most common cause of CF-related mortality [106], accounting for 3.5-7.8%[107].

The true prevalence of CFLD is difficult to estimate because the lack of sensitive and specific diagnostic tools. Based on the currently available clinical, biochemical and radiological methods the prevalence of liver disease is estimated at 26 to 45% [104;105;108]. However, these prevalence rates are likely an underestimation since, in older autopsy studies, focal biliary cirrhosis is reported in 72% of the cases [109]. Risk factors for the development of CFLD are also a matter of debate and might include male sex [108] (although not found in [105;110;111]), CFTR genotype [108;110;112] (not found in [104;105;111;113]), age at diagnosis of CF [113], age at evaluation [110;112], meconium ileus [104;108;113] (not found in [105;110-113]), severity of pulmonary disease [111;113] (not found in [104;110]), pancreatic insufficiency [104;110] and height or weight [111;113] (not found in [110]).

In modern hepatology, non invasive tools for the diagnosis of presymptomatic liver disease are needed in order to prevent, if possible, progression of fibrosis with specific medical therapies. Furthermore, there is also a continuous search for minimally invasive, sensitive and quick detection of clinically important liver disease in order to start screening for esophageal varices, hepatocellular carcinoma and to prevent other complications of chronic liver disease. Although liver biopsy remains the gold standard, limitations are considerable, including patient discomfort and rare but serious complications such as bleeding or pneumothorax and a mortality rate of 1/10,000 to 1/12,000 [114]. Moreover, only 1/50,000 of the liver volume is investigated resulting in sampling error in focally distributed liver disease as CFLD [114].

Alternative attempts to diagnose and follow-up the liver disease range from routine biochemistry and calculated scores to surrogate fibrosis markers in serum, hepatic clearance tests, various imaging techniques and more recently the use of non-invasive transient elastography (Fibroscan ®) [115].

Transient elastography is performed with an ultrasound transducer probe mounted on the axis of a vibrator. A mechanical pulse is applied directly above the liver parenchyma through an intercostal space. The pulse propagations are measured by pulse-echo acquisitions. Their velocity is directly related to the stiffness of the underlying liver tissue [116].

Although more than 55 studies have validated elastography in patients with hepatitis B, C, primary biliary cirrhosis (PBC) or sclerosing cholangitis (PSC), non-alcoholic steatohepatitis (NASH), haemochromatosis and post-transplant (for a meta-analysis see [116]), only one study included patients with cystic fibrosis (n=42) [117]. However, this study did not include a separate analysis of the results in CF patients, did not take into account the special characteristics of CFLD as the main objective was to validate the use of the Fibroscan device in children. Additionally, there are only 3 studies in the paediatric population evaluating and validating the Fibroscan in the detection of liver fibrosis [117-119]. As CFLD does develop early in life and new cases after 20 years of age are rare [104;105], this mainly paediatric age group is the most interesting for screening and diagnosing CFLD. Early diagnosis allows a timely start of ursodeoxycholic acid therapy [111] or could at least be used to monitor the evolution of CFLD.

Regarding the pathogenesis, the development of CFLD is currently explained by a biliocentric hypothesis. The CFTR-gene is normally expressed in the cholangiocytes that line the bile duct(ule)s and gall bladder, but not in the hepatocytes. The current hypothesis on the etiology of CF related liver disease (CFLD) is that by the decreased/absent cystic fibrosis conductance regulator chloride channel function the water and sodium transport to the bile diminishes. This leads to increased viscosity, diminished bile flow and increased concentrations of bile salts. This results in turn in inflammation and deposition of collagen around the bile ducts and portal tracts and to the 'characteristic' focal biliary and periportal cirrhosis which evolves to cirrhosis with portal hypertension [106].

However, there are some findings questioning this 'biliocentric' hypothesis. Histologically, bile inspissation is not common and does not correlate with the fibrosis noted. In fact, it is surprisingly infrequent [120]. Moreover, ultrastructural studies do not support the theory that

cholestasis is the pathogenetic factor in CFLD [121]. Clinically, the complications of portal hypertension (hepatosplenomegaly, variceal hemorrhage, ascites) are more frequent (up to 86% of patients with CFLD develop varices [107]) than with other types of biliary cirrhosis. The classic stigmata of biliary cirrhosis are also less prevalent (itching, jaundice, erythema palmare, spider naevi). Biochemically, the cholestasis in CF is traditionally described as minimal to moderate [107], which contrasts with a 'biliary' type of cirrhosis. Cholestasis is even not present in CFLD except in the distinct pathology of neonatal cholestasis which is unrelated to the subsequent development of CFLD [107]. Biochemical tests are poorly predictive of the severity of liver disease [106;122]. Finally, treatment of patients with CFLD with ursodeoxycholic acid, has only shown to be useful in patients with abnormal biliary drainage on scintigraphy [123]. This confirms that mechanisms other than bile duct obstruction play a role in the pathogenesis of CFLD [124]. All these clinical, biochemical and therapeutic findings suggest -at least partially- that an alternative explanation of CFLD is needed. Overall, portal hypertension is the main issue and cause of hepatic death while hepatic failure is a late event that is aggravated by bleeding episodes [107;122]. Even at the time of liver transplantation there is only a mild to moderate stage of cirrhosis [125].

Objectives of the research

1. General aims

Platelets are increasingly identified as important mediators in various diseases. We aim to better characterize platelet function in biliary (cholestatic) liver disease and in cystic fibrosis. Additionally we aim to better study a specific, biliary liver disease: cystic fibrosis associated liver disease.

2. Specific aims

1. The effect of cholestatic liver disease on platelet function.

There is conflicting data on platelet function in chronic cholestatic liver disease. It is not clear if there is hypo- or hyperaggregability. Therefore, we aim to study the loss or gain of platelet function due to a plasmatic or intrinsic platelet factor in bile-duct ligated rats.

2. The effect of cystic fibrosis on platelet function: Is it mediated by essential fatty acids?

Platelets have been implicated in the development of CF lung disease. There is conflicting data on the platelet function in CF. Additionally, a hitherto undefined plasmatic factor plays a role. This could be a decrease in essential fatty acids. Therefore, we aim to characterize platelet function and inhibition thereof in CF and study the effect of docosahexaenoic acid supplementation on inhibition of platelet function.

Essential fatty acid deficiencies are well-known in CF, however, there aetiology remains elusive. Therefore we aim to study the effect of the diseased CF lung on essential fatty acids by comparing patients that underwent a lung transplantation with age-matched CF and healthy controls.

3. The nature of cystic fibrosis associated liver disease: Are there reliable non-invasive diagnostic tests? Is it a biliary/cholestatic disease?

Currently there are no good modalities to detect or screen for CFLD. The performance of the fibroscan in this patient population remains unstudied. Therefore, we aim to evaluate the

diagnostic accuracy compared to other diagnostic tools as well as the relation of the liver stiffness to risk factors for CFLD.

The current hypothesis on the origin of CFLD suggests an important biliary component. However, CFLD is often characterized by portal hypertension rather than cholestasis. Therefore, we aim to better characterize patients with CFLD and study the histology of CFLD.

Chapter 1: ADP-degrading enzymes inhibit platelet activation in bile duct-ligated rats

Adapted from:

P Witters, M Hoylaerts, K Freson, R De Vos, J van Pelt, F Nevens, C van Geet and D Cassiman; "ADP-degrading enzymes inhibit platelet activation in bile duct-ligated rats." Journal of Thrombosis and Haemostasis: JTH, vol. 8, Feb. 2010, pp. 360-368.

Abstract

Background

The effect of cholestatic liver disease on primary haemostasis function remains ill-defined.

Objectives

To determine the platelet function and identify the mechanisms involved in the observed platelet function in cholestatic rats.

Methods

Platelet function was studied in a model of 2 week bile-duct ligation and compared to sham operation in rats with and without a storage pool defect.

Results

ADP and collagen-induced platelet aggregation were clearly impaired following bile-duct ligation ($p<0.01$ for areas under the curve). Cross-over experiments, with sham platelets in bile-duct ligated plasma and vice versa, demonstrated that this is due to inhibition by a plasmatic factor as sham platelets aggregated less in cholestatic plasma ($p<0.03$) and equal to platelets from bile-duct ligated rats when performed in the same sham or cholestatic plasma. Moreover, in bile-duct ligated rats, platelet ultrastructure was unaffected and platelet aggregation was similar to sham platelets when resuspended in the same plasma (p=NS). Aditionally, studies in storage pool deficient rats showed no role for platelet exhaustion.

This plasmatic factor causing impaired aggregation was shown to be an increased total activity of ADP-degrading enzymes upon bile-duct ligation ($p<0.01$) as there is no decreased aggregation with a stable ADP-analog in bile-duct ligated rats (p=NS vs. sham-operated). Furthermore, preincubation of plasma from bile-duct ligated rats with ADP decreased aggregation more compared to Sham plasma ($p<0.01$).

Conclusions

Bile-duct ligation does not affect the intrinsic platelet function but impairs platelet activation via release of ADP-degrading enzymes in the circulation.

Introduction

Cirrhosis and chronic liver disease are characterized by haemostatic disturbances. Abnormalities in the pro- and anti-coagulatory pathways are well-studied [1;2]. However, the primary haemostatic system (in particular platelet function) has received less attention while platelet numbers are decreased and various functional defects have been demonstrated [3].

Moreover, the effect of cholestatic liver disease on platelet function is even less clear. Some platelet function studies in cholestatic patients are, contrary to other types of liver disease, indicative of hyperaggregability. This has been suggested by thromboelastography [4] , by sonoclot analysis [5] and PFA-100 analysis [4;5]. Platelets of patients with primary biliary cirrhosis and primary sclerosing cholangitis also have increased activation markers on flow cytometry analysis (CD42b and ligand induced binding sites [4]). Similarly, platelets from bile-duct ligated (BDL) rats (a well-studied animal model of cholestatic liver disease), show an increased rise in cytosolic calcium after stimulation (the final common pathway in platelet activation)[6]. This would be consistent with increased platelet activity.

However, not all reports are equivocal on platelet hyperaggregability, which could not be demonstrated by platelet aggregometry in both patients [7]; and in animal models [8-10]. Moreover, there is a prolonged bleeding time [10] and a loss of releasable ATP [7], as well as a reduced expression of CD62P after platelet stimulation [4]; which is all indicative of decreased platelet function. As a physiologic explanation for platelet hypoaggregability, ‘platelet exhaustion’ (intravascular activation with subsequent loss of granule secretion and hence hypoaggregability when tested in vitro) has been proposed [6;7;11] and refuted [12].

The underlying mechanism for altered platelet function in cholestasis is unclear. It could be due to an intrinsic change in platelet function (e.g. increased aggregation by altered calcium homeostasis [6]) or decreased aggregation due to a storage pool deficiency [7]) or due to the effect of a plasmatic factor (e.g. inhibition by bile acids [8] or stimulation by bilirubin [8]).

Bile duct ligation is a well-known model for ‘posthepatic’ cholestasis and as it affects biliary transport systems also for secondary intrahepatic cholestasis (reduced sodium dependent bile salt uptake by hepatocytes). Upon bile duct ligation there is bile stagnation and plugging in the bile ducts. This leads to increased biliary pressure. Furthermore, due to the increased canalicular pressure and the fact that tight junctions are the only barrier between bile and portal blood, there is reflux of bile component which leads to the associated centrolobular

cholate injury. In an attempt to overcome the obstruction there is a proliferation of the bile ducts (termed ductular reaction). This leads to portal expansion and periportal fibrosis (by day 8-12 in rats) and advanced fibrosis by two weeks [13].

.The aim of our study was to clarify these conflicting data on platelet function in chronic cholestatic liver disease. Therefore, we studied the gain or loss of platelet function due to a plasmatic or intrinsic platelet factor in bile-duct ligated rats.

Materials and methods

Experimental design and animal models

Male Wistar rats and Fawn-Hooded rats weighing 250g were ordered from Janvier (Le Genest Saint Isle Cedex, France) and were divided in 2 groups: the bile-duct ligated (BDL) group and the sham-operated (Sham) group. Invasive procedures were performed under xylazine/ ketamine anesthesia, in accordance to local animal care guidelines, as described previously [14]. In the Sham-group at laparotomy, the structures were only exposed without ligation.

All animals were sacrificed at two weeks after the laparotomy and blood was taken in Vacutainer tubes (Becton-Dickinson, Erembodegem, Belgium) by direct puncture of the abdominal aorta with a 21 gauge needle.

Biochemistry

Heparin-anticoagulated blood (17 I.U./ml) was used to measure albumin, alkaline phophatases, AST, ALT, bile acids, total and direct bilirubin concentrations via standardized automated procedures. The whole blood cell count was performed on a cell-counter with rat specific parameters (Cell Dyn 2000, Abbott, Chicago, IL, USA).

Citrated blood (0.109 M citrate) was immediately centrifuged at 1,500 g and plasma was stored for thrombin-antithrombin complex analysis (Enzygnost, TAT micro, Dade Behring, Marburg, Germany), according to the manufacturers' recommendations.

Phosphatase activity

Phosphatase activity was determined on phosphate-depleted (G10 column filtrated) citrated (0.109M) plasma. Protein concentrations (Bradford assays) were performed on an aliquot before and after G10 column filtration. The G10 filtrated sample (60µL) was incubated in 180 µL buffer pH 7.4, containing 67mM TrisHCl, 2mM $CaCl_2$, 1mM $MgCl_2$, with or without 0.33 mM adenosine diphosphate (ADP, Sigma Chemical, St.Louis, USA) (final concentration). The sample was incubated on 37°C for exactly 15 minutes. The phosphatase reaction was stopped by addition of the detection dye (20 µL) giving a final concentration of 2.4 mM H_2SO_4, 8 mM sodium molybdate, 2.6 mM malachite green and 0.17% Tween 20. After 10 minutes of incubation at room temperature the photometric absorption of the sample was measured at 655nm, in comparison with a range of standard series of phosphate with or without ADP. Results were calculated as difference in phosphate production in the presence

versus absence of ADP per mg protein, (corrected for protein concentration due to G10 filtration) and expressed as mmol phosphate production per ml plasma.

Glutathione determination

Heparin-anticoagulated blood, treated with metaphosphoric acid (3.75% final concentration), was centrifuged at 4°C, 1,500g for 5 minutes. The supernatant was stored at -80°C until analysis. The glutathione content was determined according to the recycling method, as described previously [15].

Whole blood aggregation

Discarding the first 1.5 ml of blood to eliminate artefactual platelet activation, citrated blood (0.109 M citrate) was used for the whole blood aggregation within one hour of blood sampling. To this end, 250 µL of rat blood was incubated at 37°C and rotated at 900 rpm on an orbital shaker in a 24-well plate. After 1 minute 5 µL platelet agonists were added: 0, 0.85 or 1.7 µM ADP , 0, 0.625 or 1.25 ng/ml Horm collagen (Nycomed Arzenmittel, Munich, Germany) and 13, 26 or 52 nM 2-(methyl-thio-)adenosine 5-diphosphate (2-MeSADP, Sigma), final concentrations. Samples of 25 µL were removed at 1, 2 or 3 minutes (for ADP) or at 2, 3.5 or 5 minutes (for collagen). These samples and a reference sample were fixed in 1/10 diluted cell-fix (Becton Dickinson, Franklin Lakes, NJ, USA), stored for one hour on ice. Platelets were counted in these samples and the percentage platelet aggregation was calculated as [(number of platelets in reference sample)-(number of platelets in the sample)] / (number of platelets in reference sample) x 100%. Amplitudes (% aggregation) and areas under the curve (AUC) were calculated.

To test the influence of cholic acid (Calbiochem, Darmstadt, Germany), reduced glutathione (GSH) (INC Biomedicals inc., Ohio, USA) and apyrase (Sigma) these agents (in 12.5µL aliquots in sodium chloride 0.9% at neutral pH) were pre-incubated with whole blood at least 2 minutes before the aggregation or 30 seconds in case of hepatocyte growth factor (HGF) (R&D systems, Minneapolis, USA). Cholic acid was used at final concentrations of 25, 50 and 100 µg/ml; HGF at a final concentration of 50ng/ml; GSH at a final concentration of 10, 50 or 100 µM and apyrase at a final concentration of 0.5U/ml. In reference conditions without these agents, sodium chloride 0.9 % was added in similar aliquots.

Optical aggregometry

In the cross-over experiments, citrated blood from Sham and BDL rats was pooled in each experiment, when necessary, to obtain sufficient amounts of platelet rich (PRP) and platelet poor plasma (PPP). PRP was prepared from this blood by differential centrifugation and the platelet count was adjusted with PPP to 250-300x10^9 platelets/l. Platelets from BDL or Sham rats were at least 5-fold diluted in BDL or Sham PPP and vice versa. Aggregation was performed on two dual-channel Chrono-Log aggregometers (Chronolog Corp., Philadelphia, USA) after activation with ADP (5 µM and10 µM, final concentration).

In the pre-incubation experiments, Sham PRP was diluted to 250-300x10^9 platelets with BDL or Sham PPP and stimulated with 10µM ADP final concentration either preincubated in the plasma for 10 minutes or directly into the aggregation.

Results are expressed as relative AUC (the most sensitive variable in ADP stimulation [16]) compared to the control condition (sham platelets in sham plasma).

Histology

The liver was fixed in formol 6%. Samples were then embedded in paraffin, sectioned (7µm), mounted on slides and stained with haematoxylin and eosin or sirius red. On sirius red slides (collagen staining), image analysis was performed using Analysis D (Microsuite 5, Olympus America Inc., Pennsylvania, USA). Seven or more fields (10 x) were analyzed to determine the average surface stained red (Average % per field) for each animal.

Platelet electron microscopy was performed as described previously [17].

Statistical Analysis

SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA) was used. All results are expressed as means ± standard deviation. For differences between two groups, the Mann-Whitney U test was used. To compare multiple groups, the Kruskal-Wallis test was used. To compare related samples, the Wilcoxon signed rank test was used. A p-value <0.05 was considered statistically significant.

Results

Animal models: general characteristics

Two weeks after BDL, the Wistar rats became ill and had lost weight. The BDL-group demonstrated clear cholestasis and deranged liver tests, compared to the Sham-group (Table 1.1). On liver histology the BDL-group showed prominent ductular reaction and advanced fibrosis (Table 1.1).

Table 1.1: Characteristics at sacrifice of the different experimental groups

	Wistar rats		Fawn-Hooded rats	
	BDL	Sham	BDL	Sham
Body weight (g)	269 ± 25 ^{c}	367 ± 26	215 ± 34 ^{c}	287 ± 6.8
AST (U/L)	235.44 ± 127.29 ^{aa}	79.50 ± 5.42 ^{aa}	353.17 ± 202.17 ^{bb}	68.50 ± 4.50 ^{bb}
ALT (U/L)	92.67 ± 41.47 ^{a}	59.25 ± 12.75 ^{a}	96.50 ± 22.01 ^{bb}	52.50 ± 6.60 ^{bb}
Total bilirubin (mg/dL)	11.22 ± 4.70 ^{aa}	0.01 ± 0.04 ^{aa}	8.22 ± 3.95 ^{bb}	0.00 ± 0.00 ^{bb}
Direct bilirubin (mg/dL)	8.89 ± 4.34 ^{aa}	0.00 ± 0.00 ^{aa}	6.41 ± 3.15 ^{bb}	0.00 ± 0.00 ^{bb}
Alkaline phosphatase (U/L)	896.22 ± 164.55 ^{aa}	509.75 ± 187.97 ^{aa}	895.20 ± 61.86 ^{b}	621.25 ± 55.39 ^{b}
Albumin (g/L)	32.23 ± 2.61 ^{aa}	38.18 ± 3.12 ^{aa}	31.11 ± 3.41 ^{bb}	42.25 ± 1.55 ^{bb}
Total bile acids (µM)	88.44 ± 40.14 ^{aa}	5.51 ± 6.07 ^{aa}	nd	nd
Sirius red stain (%)	6.58 ± 2.98 ^{aa}	0.65 ± 0.36 ^{aa}	nd	nd
Platelet count (*10³/µL)	881.55 ± 154.23 ^{c}	936.25 ± 205.28	661.00 ± 147.31	831.00 ± 123.61 ^{c}
Leucocyte count (*10³/µL)	27.15 ± 10.60 ^{a,cc}	14.06 ± 7.67 ^{a}	8.78 ± 6.13 ^{b}	2.37 ± 0.44 ^{b,cc}
Thrombin-antithrombin complexes (pg/ml)	4.63 ± 2.01	2.51 ± 1.38	nd	nd

nd= not determined, ^{a} p< 0.05 and ^{aa} p<0.01: BDL Wistar versus Sham Wistar,
^{b} p< 0.05 and ^{bb} p<0.01: BDL Fawn-Hooded versus Sham Fawn-hooded,
^{c} p< 0.05 and ^{cc} p<0.01: BDL Wistar versus BDL Fawn-Hooded
n is at least 4 in every condition.

Platelet number and function after bile-duct ligation

Platelet counts were not different between the two groups (Table 1.1). There was also no activation of the coagulation system (normal thrombin-antithrombin complex levels, Table 1.1). Platelet aggregation by ADP in whole blood was reversible; being maximal after one minute, followed by desaggregation. In the BDL group (n= 11), after stimulation with 0.8 µM or 1.7 µM ADP, aggregation was impaired at all measured time points compared to the Sham group (n=11) (Figure 1.1 a). Consequently, the AUC was significantly decreased in the BDL group, after stimulation with 0.8 µM ADP (69 ± 29m% versus 145 ± 35m%, $p<0.01$) and 1.7 µM ADP (105 ± 38 m% versus 223 ± 21 m%, $p<0.01$).

Stimulation with collagen induced progressively more aggregation without desaggregation. At 3.5 and 5 minutes after 0.62 ng/mL collagen stimulation and at all time points with 1.25 ng/mL collagen, a hypoaggregability in the BDL group (n=11) versus the Sham group (n=11) was observed (Figure 1.1 b). The AUC was significantly smaller in the BDL group after stimulation with collagen 0.62 ng/ml (140 ± 105 m% versus 309 ± 107m%, $p<0.01$) and with collagen 1.25 ng/ml (265 ± 145 m%, versus 387 ± 28 m%, $p<0.01$).

Figure 1.1 a: ADP-induced whole blood aggregation in BDL and Sham Wistar rats
Mean amplitudes plus standard deviation are shown. N=11 in each condition, *=p<0.05 and **=p<0.01

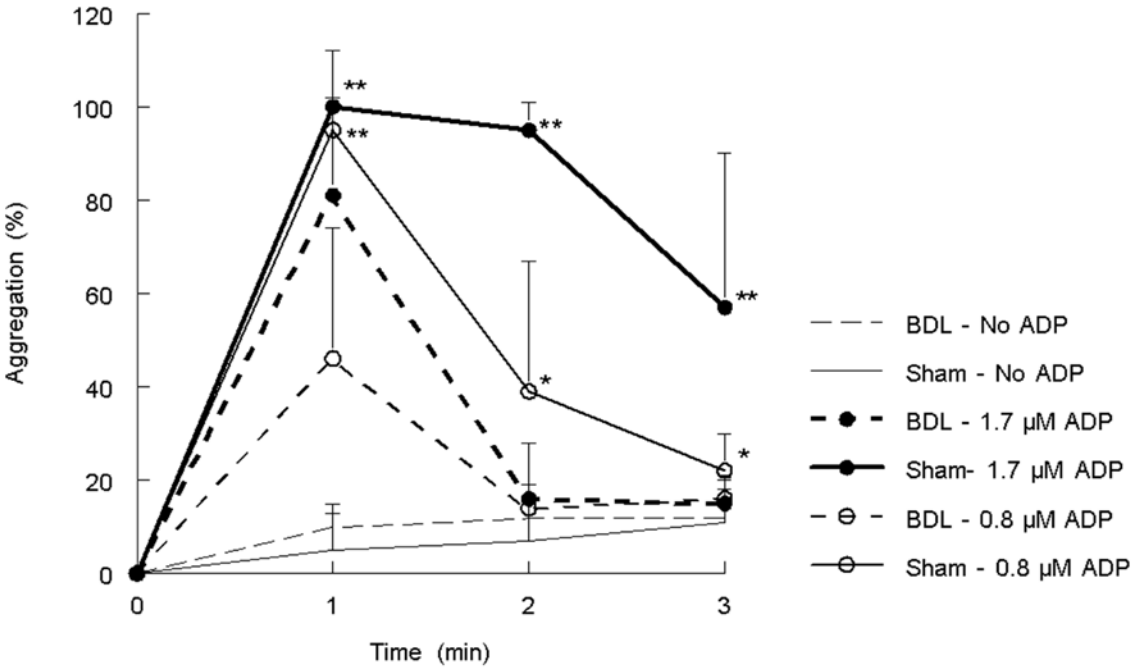
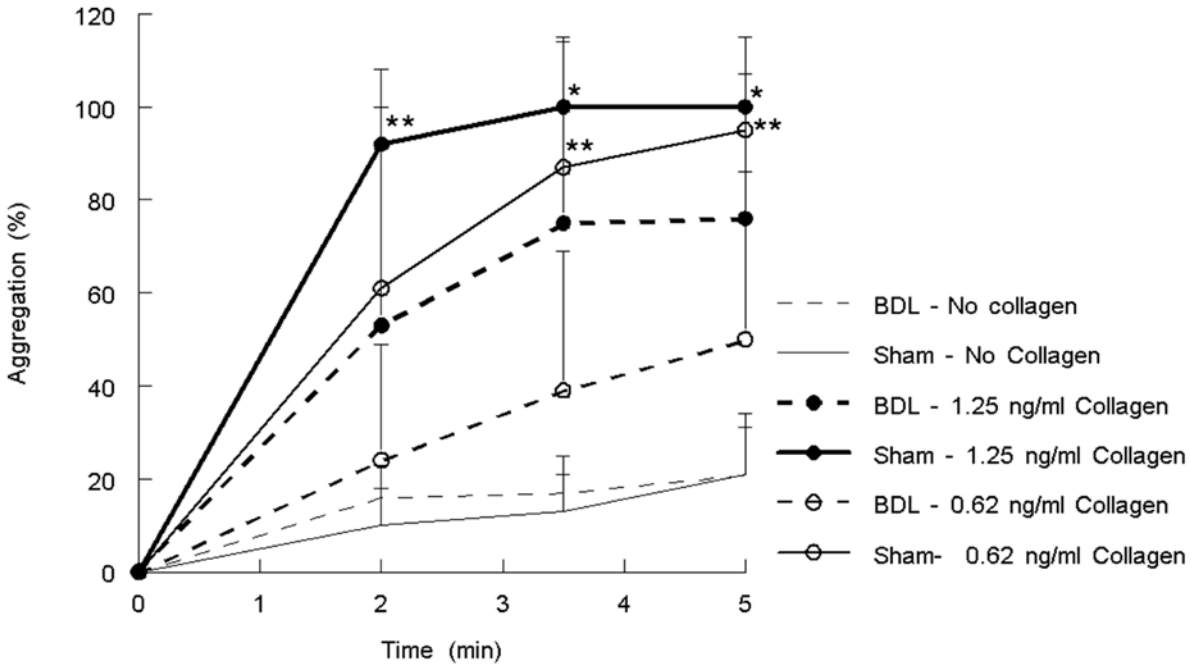


Figure 1.1 b: Collagen-induced whole blood aggregation in BDL and Sham Wistar rats
Mean amplitudes plus standard deviation are shown. N=11 in each condition, *=p<0.05 and **=p<0.01



Platelet ultrastructure: electron microscopy

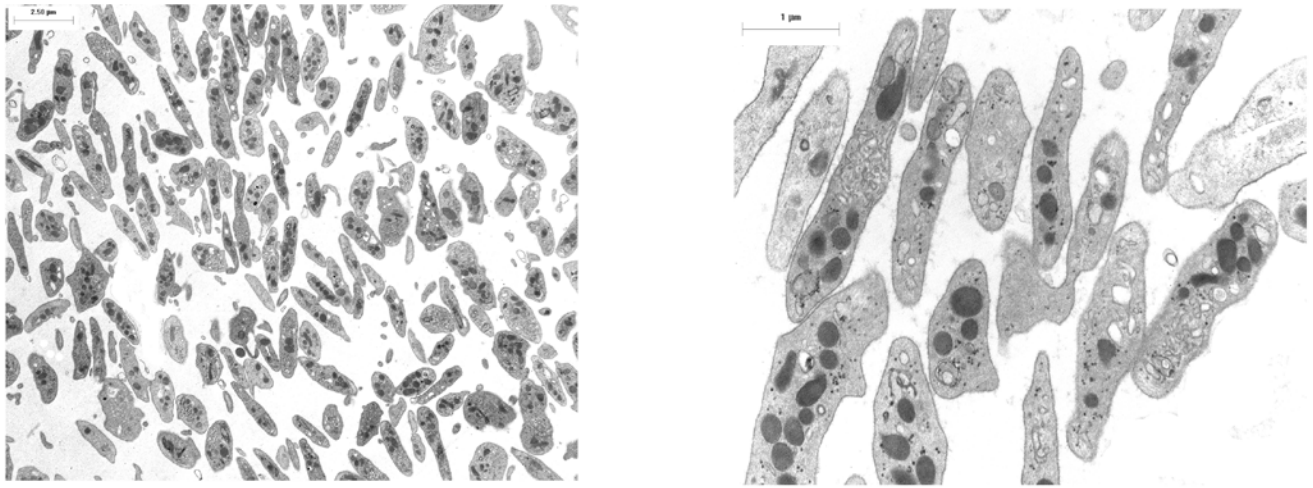
To investigate whether the loss of platelet function would result from ultrastructural platelet abnormalities, we performed electron microscopy on platelets of BDL rats (n=6) and Sham rats (n=6). However, we found no differences in platelet ultrastructure (size, general appearance, type and number of granules, granule content), between these two groups (Figure 1.2 for representative images).

Storage pool deficiency: studies in Fawn-hooded rats

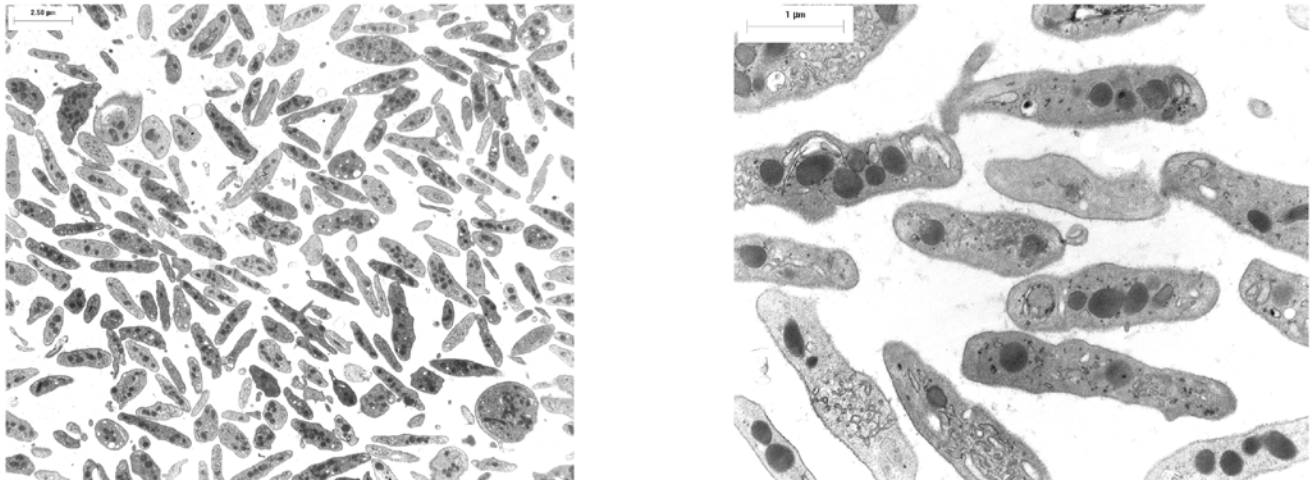
To investigate whether loss of platelet function would result from platelet exhaustion or degranulation, we studied platelet function in a storage pool deficiency model which we submitted to bile-duct ligation. Therefore, we performed platelet aggregation studies in Fawn-hooded rats, known for their total lack of collagen-induced ATP-secretion [18]. Upon bile-duct ligation, Fawn-Hooded rats undergo similar hepatic changes as Wistar rats (Table 1.1).

Figure 1.2: Electron micrographs of platelets

a) Sham group: overview (left) and detail (right) of platelets (representative pictures)



b) BDL group: overview (left) and detail (right) of platelets (representative pictures)



After stimulation with ADP, aggregation was impaired in the BDL group (n=6) compared to the Sham group (n=4) (Figure 1.3 a). This results in a significantly decreased AUC after stimulation with 0.8µM ADP (20 ± 9 m% versus 115 ± 16 m%, p< 0.01) and after stimulation with 1.7µM ADP (66 ± 27 m% versus 187 ± 22 m%, p< 0.01). As the difference in

aggregation in the BDL versus Sham group remained the same in the Fawn-Hooded rats compared to the Wistar rats, the aggregation defect in the BDL groups cannot be attributed to a secretion defect. After stimulation with collagen at the low doses used in present work, no aggregation in Fawn-Hooded rat platelets was observed (Figure 1.3 b), as expected.

Influence of plasmatic factor on platelet aggregation

To study the possibility of inhibition of platelet aggregation by plasmatic factors, we performed aggregations by optical aggregometry in platelet rich plasma. In a cross-over design, platelets of the Sham group or BDL group were strongly diluted in Sham or BDL plasma and vice versa. In agreement with the whole blood aggregation studies, the aggregation response after stimulation with 5µM ADP and 10µM ADP of BDL platelets in BDL plasma was significantly lower compared to the response of Sham platelets in Sham plasma (42 ± 9 % versus 100%, p= 0.03 and 50 ± 5%, versus 100%, p<0.01, results expressed as AUC relative to the Sham platelets in Sham plasma reference).

When comparing the different groups (Figure 1.4), there were significant differences in relative AUC after stimulation with ADP (p=0.029). Sham platelets aggregated less upon suspension in BDL plasma, after stimulation with 5µM ADP (p=0.03) and 10µM ADP (p<0.01) compared with their aggregation in Sham plasma. Similarly, BDL platelets aggregated better when suspended in Sham plasma than in BDL plasma after stimulation with 10 µM ADP (p=0.03). With 5µM ADP, only a trend towards the same effect was seen (p=0.08). On the contrary, there was no difference in Sham or BDL platelets when suspended in the same plasma (Sham or BDL) after stimulation with 5 or 10 µM ADP (p= NS, Figure 1.4). Finally, BDL platelets aggregated better in Sham plasma than Sham platelets aggregate in BDL plasma after stimulation with 10µM ADP (p<0.01). These findings excluded an intrinsic platelet dysfunction.

Figure 1.3 a: ADP-induced whole blood aggregation in BDL and Sham Fawn-Hooded rats
Mean amplitudes plus standard deviation are shown. N=6 in the BDL group, n=4 in the Sham group and **=p<0.01 .

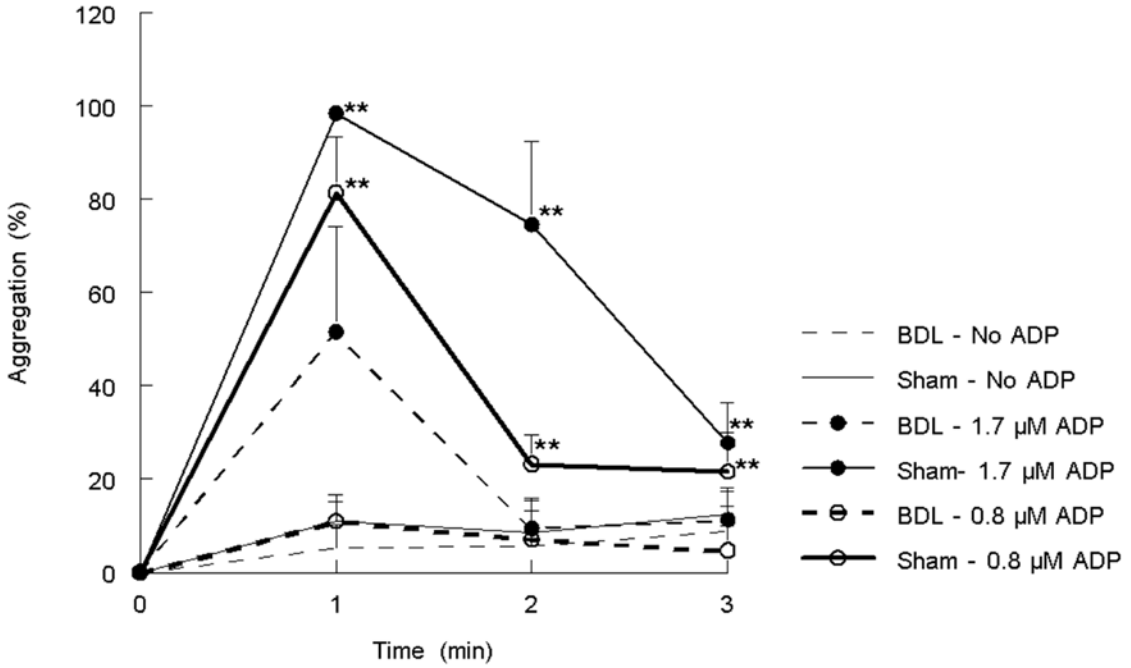


Figure 1.3 b: Collagen-induced whole blood aggregation in BDL and Sham Fawn-Hooded rats
Mean amplitudes plus standard deviation are shown. N=6 in the BDL group, n=4 in the Sham group. p=not significant at all time points

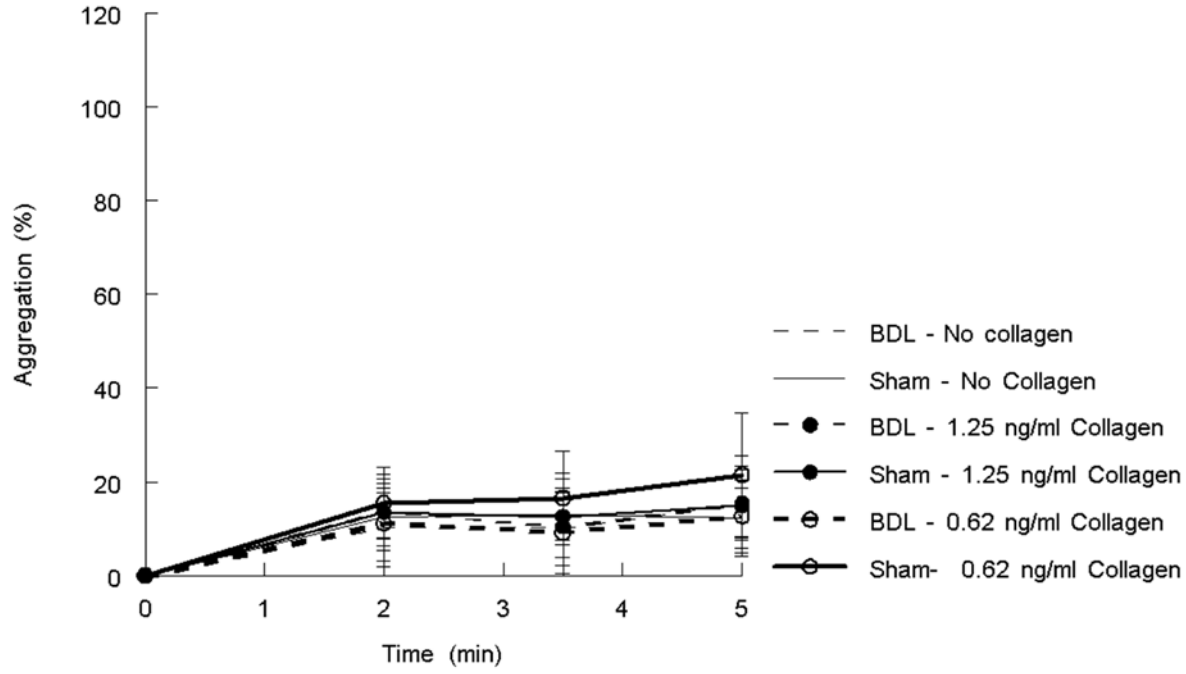
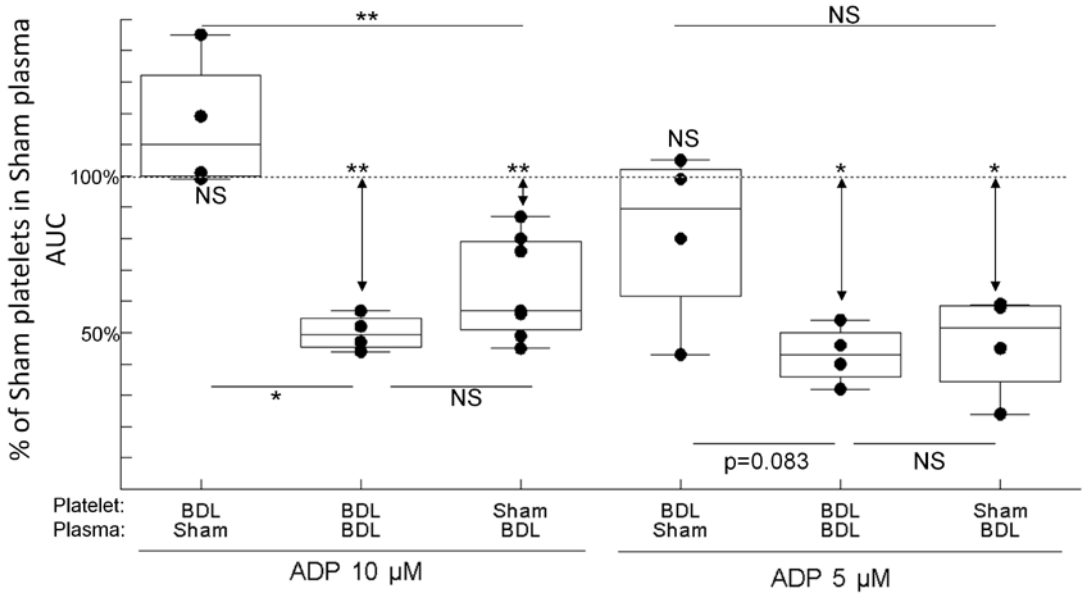


Figure 1.4: Crossover Experiment summary
Each dot represents an individual measurement (at least 4 experiments per condition).
*=p<0.05 and **=p<0.01 compared to the sham platelets sham plasma reference (dashed line) or between the groups indicated.



Study of candidate plasmatic factors reducing platelet aggregation in cholestatic liver disease

Our next aim was to determine the responsible plasmatic factor that impairs ADP induced platelet aggregation.

Bile salts were significantly increased upon bile-duct ligation (88.44 ± 40.14 µM (n=6) versus 5.51 ± 6.07 µM (n=6), p=0.003). However, pre-incubation of whole blood (of Sham rats) with cholic acid 25-100µg/ml (the pathologically attainable concentrations [19]) did not result in any difference in whole blood aggregation with ADP 0.8 or 1.7 µM or with 0.62 or 1.25ng/ml collagen (p=NS for all time points and AUC's).

Hepatocyte growth factor was not significantly elevated in our series of BDL rats compared to Sham operated animals (0.44 ± 0.18 ng/ml (n=6) versus 0.29 ± 0.12 ng/ml (n=5), p=NS). Moreover, the addition of HGF 30 seconds before the addition of an agonist (ADP 1.7µM or 1.25ng/ml Collagen) in Sham rats did not result in any inhibition of aggregation (p=NS for all time points and AUC's).

The GSH level was decreased in our series of BDL rats compared to the Sham group (7.79 ± 3.48 µM versus 21.74 ± 4.67µM, $p<0.01$). However, the addition of 10-100µM GSH to the whole blood of BDL rats (to restore GSH deficiency) in the whole blood aggregation assay did not result in a change in aggregability (p= NS for all time points and AUC's).

We then hypothesized the functional aggregation deficit could be due to the presence of **enzymes that degrade ADP** as it is well-known that alkaline phosphatase and 5'-nucleotidases increase in cholestatic patients and are capable of degrading the agonist ADP to the antagonist adenosine. Indeed alkaline phosphatase levels were increased in our BDL rats (896 ± 165 U/l versus 510 ± 188 U/l, $p<0.01$, see table 1.1). We also measured the activity of all ADP-degrading enzymes present in the BDL and Sham plasma. BDL plasma induced more formation of inorganic phosphate per ml plasma after the addition of ADP compared to Sham plasma (46.88 ± 24.59 mmol/ml plasma versus 16.90 ± 13.55 mmol/ml plasma, p= 0.03, n=5 in each group).

We therefore performed whole blood aggregations with the stable ADP analog: 2-MeSADP. Stimulation of whole blood of BDL-rats or Sham-rats with 13, 26 or 52 nM 2-MeSADP resulted in equal aggregation responses (same amplitudes at any time point and same AUC's, Figure 1.5).

In order to show that enzymatic agonist degradation was responsible for the decreased platelet aggregation in response to ADP stimulation, we then performed pre-incubation experiments of ADP in plasma. Using optical aggregometry, the aggregatory response of sham platelets to stimulation with 10µM ADP with or without 10 minutes pre-incubation in Sham or BDL plasma was compared. As shown in figure 1.6 and previously, Sham platelets aggregated less if suspended in BDL plasma. Pre-incubation of BDL plasma with ADP abrogated the aggregatory response to ADP completely. Pre-incubation of Sham plasma with ADP also decreased this response however to a lesser extent than BDL plasma (Figure 1.6, $p<0.01$).

Figure 1.5: Effect of 2-MeSADP stimulation on Sham and BDL blood.
N≥ 3 in each condition. For simplicity only means are represented. P=NS for all the amplitudes at the different time points between the conditions with equal amounts of 2-MeSADP (BDL versus Sham).

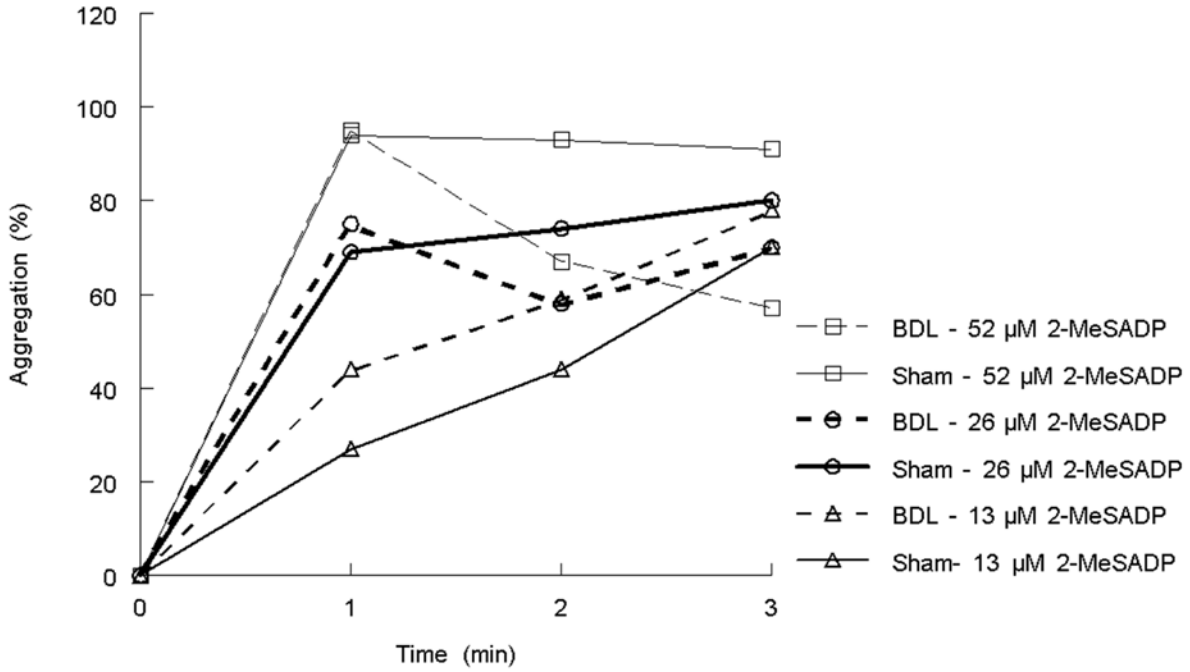


Figure 1.6: Effect of ADP-induced and ADP-pre-incubation-induced aggregation
Each dot represents an individual measurement. **=p<0.01 compared to the sham PRP in Sham plasma reference with direct addition of ADP (dashed line) or between the groups indicated.

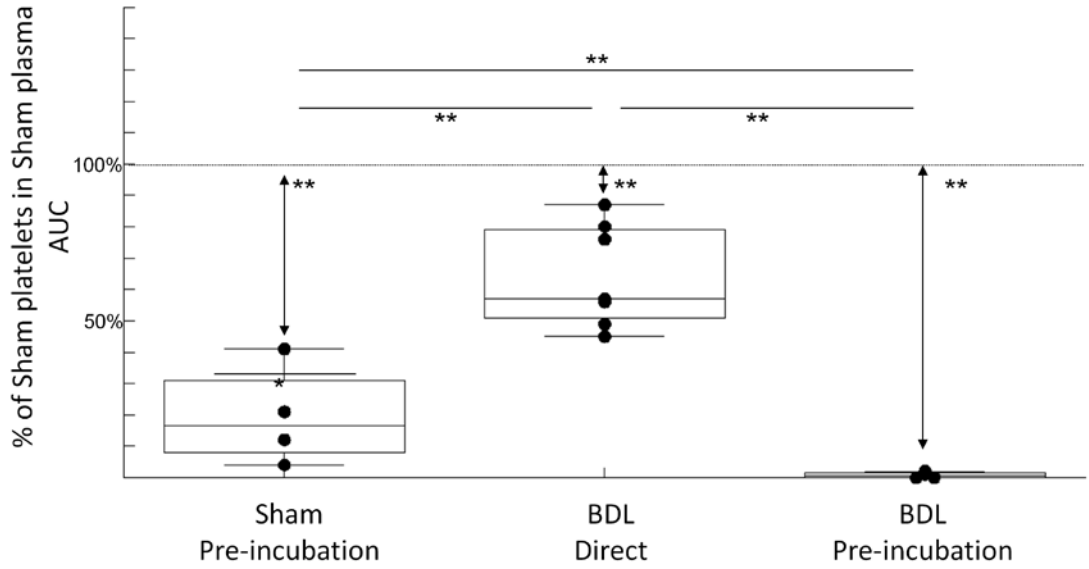
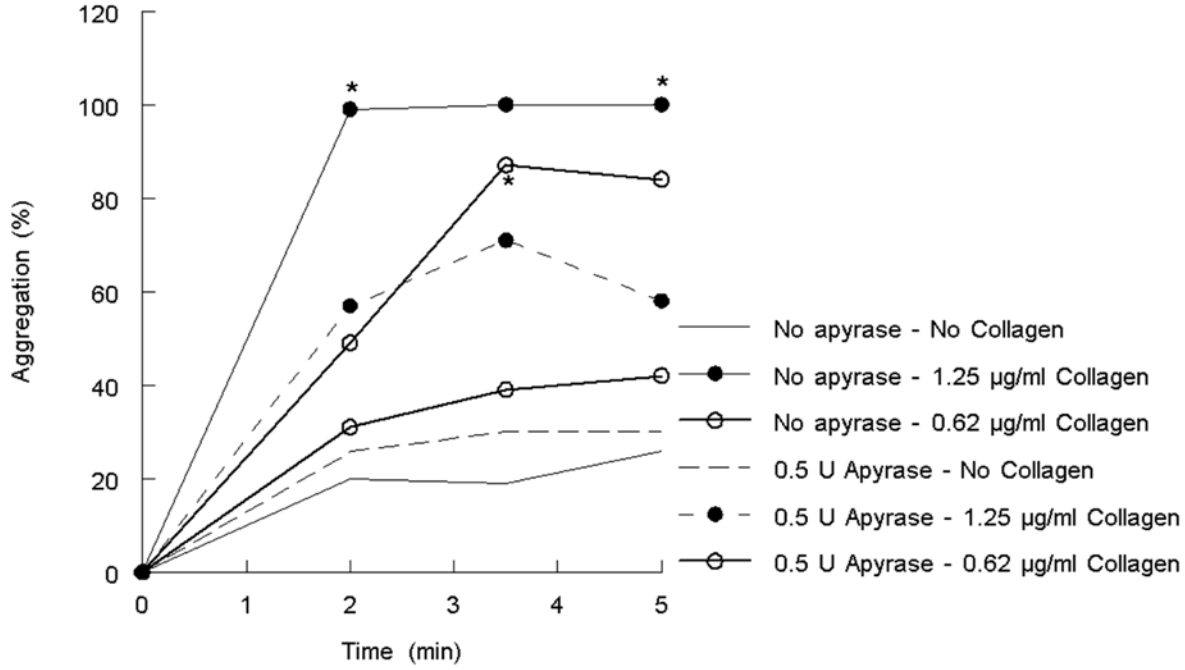


Figure 1.7: Effect of ADP-degrading enzymes (apyrase) on collagen-induced whole blood aggregation

N=5 in each condition. For simplicity only means are represented. *p<0.05 between the conditions with and without 0.5 mU apyrase and the same concentration of collagen.



Finally, to test if this inhibitory mechanism could also be relevant to the collagen induced aggregation we preformed whole blood aggregations with collagen in the presence of 0.5 U/ml apyrase (an ADP-degrading enzyme). Whole blood aggregation with 0.62 ng/ml and 1.25 ng/ml collagen was inhibited by apyrase (Figure 1.7). There was a significant decrease in AUC after stimulation with 0.62 ng/ml collagen (422 ± 173m% (n=5) versus 843 ± 390m% (n=5), p=0.04) and with 1.25 ng/ml (697 ± 345m% (n=5) versus 1088 ± 356m% (n=5), p=0.04). This confers that at this dose of collagen, collagen-induced aggregation is dependent on ADP secretion.

Discussion

This study shows that in 2 week BDL rats, a model of chronic cholestatic liver disease, platelet aggregation is impaired. This is not due to an intrinsic platelet defect as there is normal platelet function when platelets are studied in Sham plasma, but due to an inhibitory plasmatic factor. The lack of a difference in aggregability between the two groups after stimulation with a stable ADP analog suggested the involvement of significantly elevated levels of enzymes that degrade ADP. ADP is an important secondary agonist for platelets, also playing a role in the aggregatory response to other agonists such as collagen. Furthermore, pre-incubation of ADP in BDL plasma abrogated the aggregation, probably due to enzymatic ADP degradation.

Using whole blood and optical aggregometry we confirm the decreased aggregation of 'cholestatic platelets in cholestatic plasma' [7-10]. However, upon suspension of BDL platelets in Sham plasma, we could not detect any difference in aggregability. Previously, two studies have shown that there is indeed influence of cholestatic plasma on platelets: in patients with cirrhosis an undefined plasmatic factor sensitive to freezing has been shown to alter calcium homeostasis [20] and in bile-duct-ligated horses an inhibitory effect of cholestatic plasma on aggregation was seen [8]. However, we are the first to show that platelets of cholestatic animals aggregate normally when suspended in normal plasma. This excludes an intrinsic platelet defect.

The hypothesis of 'platelet exhaustion' [6;7;11], as underlying mechanism, was further excluded by the normal platelet ultrastructure, the lack of increased thrombin-antithrombine levels and our data describing bile-duct ligations in a rat model of storage pool disease [18]. As the latter resulted in the same aggregatory deficit in BDL platelets compared to Sham, loss of granules is not involved in their platelet dysfunction.

As a plasmatic factor is responsible for the impaired aggregation, our next aim was to identify this factor. From literature research, three candidates emerged: bile acids [8;21], HGF [22] and GSH [23]. However, upon preincubation of whole blood with bile acids, HGF or GSH at pathophysiologically relevant concentrations we could not detect any effect on the aggregation. On the other hand, our BDL rats had increased levels of alkaline phosphatase and of total ADP-degrading enzyme activity. Aggregations with 2-MeSADP (a non-hydrolysable ADP-analog) instead of ADP showed no difference between aggregations in BDL rats and Sham rats. To further show that ADP degradation plays a role, we surmised that

pre-incubation of ADP with cholestatic plasma would abrogate the aggregation. Indeed, we found that 10 minute pre-incubation of BDL plasma with ADP prevented the aggregation completely in contrast to immediate addition of ADP in the aggregation. Incubation with Sham plasma resulted in a significantly smaller decrease in aggregation.

A whole series of nucleotide degrading enzymes has been described. These include diadenosine polyphosphate hydrolases, the CD39/ecto-nucleoside triphosphate diphosphohydrolase family, nucleotide pyrophosphatases/phosphodiesterases, 5'ecto-nucleotidases, alkaline phosphatases, NAD-glycohydrolase, CD38 and adenosine deaminase [24]. Which of all these nucleotide degrading enzymes or combinations thereof is responsible for the decreased aggregation remains unstudied. Ectonucleotidases have shown to modulate platelet activation and thrombus formation [25;26]. Indeed, previously it was shown that leucocyte bound CD39 is important in ADP degradation [27] and leucocyte counts are indeed raised in BDL rats. However, since the difference in aggregation between BDL and sham rats remains when studied in PRP, other enzymes are probably also important. Characteristic of cholestatic liver disease is the increase of alkaline phosphatase and 5' nucleotidase levels which can degrade ATP, ADP and AMP [28]. Bile salts are required in cholestatic liver disease, to increase serum alkaline phosphatase activity and 5' ectonucleotidase activity, both in rats [28] and humans [29].

Interestingly, cholic acid feeding of normal rats during 4 days did also result in an impairment of aggregation [9], accompanied by a rise in bile acid levels which did suggest a direct effect of the bile acids. However, as it is known that 1% cholic acid feeding causes necrosis and cholestasis, subsequent release of ADP-degrading enzymes is possible [30]. This could explain why similar changes (inhibition of platelet activation) are seen during cholic acid feeding as following BDL.

The effect of the defect in ADP-induced aggregation we describe here, is probably also important in vivo. ADP is present in high concentration in dense granules and hence plays a role in aggregation by all agonists (cfr. collagen induced aggregation in our study). Patients with congenital defects in the P2Y12 ADP receptor show a severe bleeding phenotype [31] and ADP receptors are important targets for highly effective antithrombotic agents (as are ticlopidine and clopidogrel).

If these findings would be confirmed in humans with cholestatic liver disease, therapies targeting ADP-degrading enzymes could be expected to improve primary haemostasis,

making invasive procedures (e.g. biopsies, transplantation, endoscopic retrograde cholangio-pancreaticography) more safe or feasible. On the other hand, platelet transfusions in these patients would not have the intended effect if the platelets are inhibited by the ADP-degrading enzymes.

To conclude, we have shown, that intrinsic platelet function of BDL platelets is normal if studied in Sham plasma. The whole BDL blood platelet function is decreased due to the presence of a plasmatic factor. This factor has been identified as enzymes that degrades ADP.

Acknowledgements

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Addendum

Having demonstrated the role of cholestatic liver disease on platelets, one could wonder if there would be a similar effect on ADP-induced aggregation in other, non-cholestatic models of chronic liver disease.

In order to respond to this question, we also did whole blood ADP-induced aggregation studies in thioacetamide intoxicated rats, another model of chronic liver disease in rats [1].

Materials and methods

Male Wistar rats, weighing 200–250 g, were intoxicated with thioacetamide (TAA, Sigma, Bornem, Belgium) in drinking water (TAA-group, n=7). The TAA concentration was adapted weekly to changes in body weight, leading to liver fibrosis after 12 weeks of treatment as described previously [1]. Healthy rats weighing 230-280g served as control (Control-group, n=7).

Results

During 12 weeks of TAA intoxication, animals also became progressively ill, but without any mortality and less elevated liver tests. Cholestasis was absent (as direct bilirubin or alkaline phosphatases were normal), but fibrosis in the TAA-group was comparable to that in the BDL-group (Table 1.2).

Table 1.2: Characteristics at sacrifice of the different experimental groups

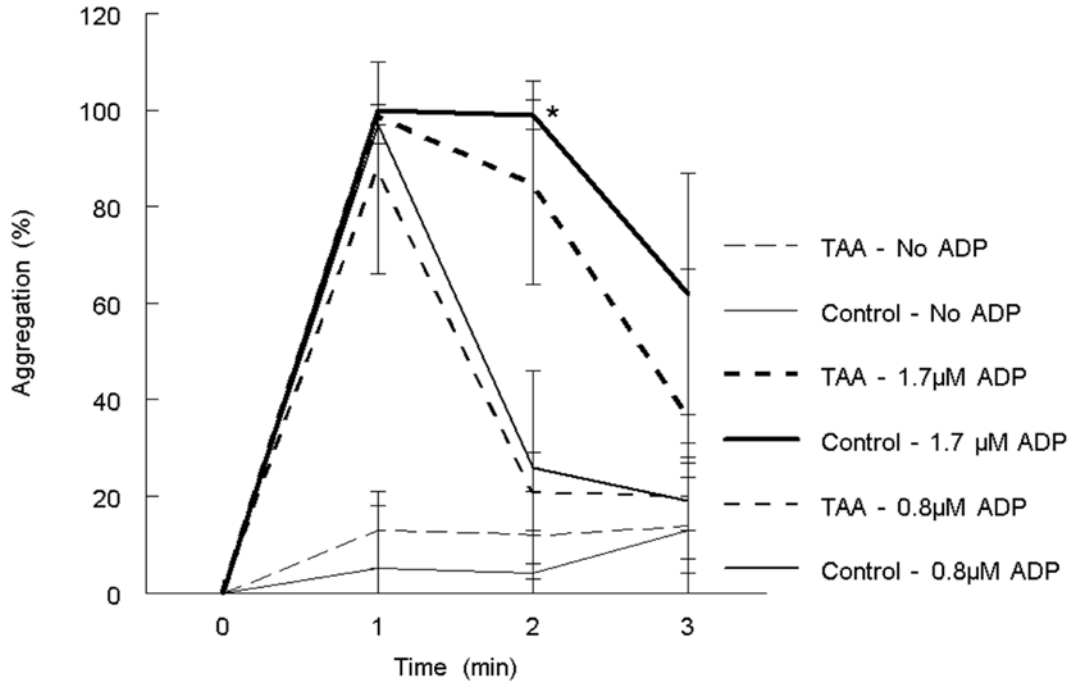
	BDL	Sham	TAA	Control
Body weight (g)	269 ± 25 [aa]	367 ± 26	275 ± 31	246 ± 16
AST (U/L)	240.5 ± 121.1 [aa, cc]	82.6 ± 10.5	111.7 ± 28.4 [cc]	91.3 ± 21.0
ALT (U/L)	93.8 ± 39.3 [a]	61.2 ± 13.3	69.1 ± 19.2	56.6 ± 20.1
Total bilirubin (mg/dL)	12.0 ± 4.6 [aa, cc]	0.02 ± 0.05	0.3 ± 0.2 [b, cc]	0.05 ± 0.09
Direct bilirubin (mg/dL)	9.6 ± 4.2 [aa, cc]	0.001 ± 0.003	0.06 ± 0.11 [cc]	0 ± 0
Alkaline phosphatase (U/L)	911.2 ± 162.2 [aa, c]	516 ± 176	753 ± 180 [c]	744 ± 1.35
Albumin (g/L)	32.1 ± 3.73 [aa, cc]	38.3 ± 3.0	40.8 ± 2.1 [cc]	42.6 ± 103
Sirius red stain (%)	6.58 ± 2.98 [aa]	0.65 ± 0.36	6.27 ± 2.28 [bb]	0.49 ± 0.16
Platelet count (*10³/µL)	970 ± 248	959 ± 166	839 ± 230	873 ± 102
White cell count (*10³/µL)	30.4 ± 10.9 [aa cc]	17.2 ± 8.0	9.9 ± 2.9 [cc]	12.5 ± 3.3
Neutrophil count (%)	38.1 ± 8.6 [aa]	14.7 ± 4.1	40.56 ± 8.26 [bb]	12.03 ± 5.66
Thrombin-antithrombin complexes (pg/ml)	4.63 ± 2.01	2.51 ± 1.38	5.07 ± 3.44	2.76 ± 2.70

nd= not determined, [a] p< 0.05 and [aa] p<0.01: BDL versus Sham, [b] p< 0.05 and [bb] p<0.01: TAA versus Control, [c] p< 0.05 and [cc] p<0.01: BDL versus TAA

On the whole blood count on day 14 there was no difference in platelet count between the TAA and control group.

There was no difference in thrombin-antithrombin levels, reflecting activation of coagulation, between BDL, Sham, TAA or controls (Table 1.2).

Figure 1.8 : ADP-induced whole blood aggregation in TAA and Control Wistar rats
Mean amplitudes plus standard deviation are shown. N=7 in each condition, *=p<0.05



In the TAA-group the ADP-induced aggregation was only significantly decreased at 2 minutes after stimulation with 1.7µM ADP (84.9 ± 21.0 % versus 98.8 ± 2.9 % in controls, p=0.033; see figure 1.8). The AUC after stimulation with 0.8µM ADP and 1.7µM was not significantly different from the control condition (119.5 ± 32.2m% versus 133.2 ± 25.9m%, p= 0.63 and 202.9 ± 34.0 m% versus 229.9 ± 14.8 m%, p=0.078 respectively).

On the other hand, the ADP-induced aggregation in TAA is significantly better than in the BDL model (maximal aggregation with 0.8 µM ADP p=0.025, and with 1.7 µM p=0.049 and AUC after stimulation with 0.8µM ADP, p= 0.025 and with 1.7µM ADP p= 0.007; Figures 1.1 a and 1.8).

Discussion

These results indicate that ADP degradation could play a role in other liver diseases in explaining impaired platelet aggregation. However, its role is less important than in the BDL model. This adds to the importance of cholestasis-induced ADP degrading enzymes in the BDL model.

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Chapter 2: DHA inhibits platelet function and can play a role in platelet hyperactivity in CF via cAMP-independent pathways

Adapted from:

Witters P, Freson K, Hoylaerts M, Thys C, Proesmans M, Vermeulen F, Dupont L, De Boeck K, Cassiman D and Van Geet C; "DHA inhibits platelet function and can play a role in platelet hyperactivity in CF via cAMP-independent pathways." Manuscript submitted to Blood.

Abstract

Background:

Cystic fibrosis (CF) is a devastating disease. Platelets and essential fatty acids (EFA) are increasingly recognized as being important players in the pathophysiology.

Objectives:

To study platelet function in CF and the influence of EFA, and more specific docosahexaenoic acid (DHA), on platelet function in vitro and in vivo.

Patients/Methods:

Platelet function tests, with an emphasis on inhibition of this function by the Gs pathway, were performed in CF patients and healthy controls before and after supplementation with DHA.

Results:

Platelets in CF displayed normal aggregation to epinephrine and collagen, normal ATP secretion and normal shear-induced activation. However, there was a decrease in inhibition of collagen-induced aggregation with prostaglandin E1 or prostacyclin. This was not mediated via cAMP or protein kinase A (PKA) activity.

DHA levels were decreased in CF patients. Addition of DHA in vitro increased the inhibition by prostaglandin E1. Likewise, a 10 day supplementation of healthy volunteers with DHA increased inhibition by prostacyclin and prostaglandin E1 without affecting collagen-induced aggregation. This was also not mediated via cAMP or PKA.

Conclusions:

DHA, a plasma factor deficient in CF, can increase inhibition of platelet function. This could be responsible for decreased platelet inhibition in CF. These findings suggest DHA supplementation could normalize platelet function in CF.

Introduction

Cystic fibrosis (CF) is the most frequent lethal genetic disorder, within the Caucasian race, with an incidence of 1 to 2500-4000. Due to the current medical treatment the survival of CF patients rose to more than 35 years, coming from less than 6 years in the 50's. Pulmonary problems are the main cause of death.

Recently, platelets have been suggested to contribute to the CF pulmonary inflammation and tissue destruction and it was hypothesized that platelet activation is an important event in CF lung disease [1;2]. Platelets are increasingly recognized as inflammatory agents: they undergo chemotaxis, release proinflammatory molecules and activate other inflammatory cells [1;3;4].

In physiological circumstances, platelet activation is counterbalanced by platelet inhibition in order to prevent a generalized prothrombotic state. This is among others controlled by the endothelium that produces eicosanoids (prostacyclins and prostaglandins) which act locally on the platelets. Thereby a signaling cascade is activated via a Gs coupled receptor that leads to the generation of cAMP and the activation of protein kinase A (PKA). This phosphorylates numerous targets which eventually lead to inhibition of platelet activation [5].

It is not clear whether platelets from CF patients demonstrate in vitro hyperaggregability or not. Hyperaggregability has been demonstrated by various authors [6-8], but could not be confirmed by others [4;9]. Nevertheless, abnormalities in the inhibition by eicosanoids have been demonstrated. Prostaglandin E_1 (PGE_1) is less capable of inhibiting ADP-induced platelet aggregation [10] and P-selectin expression (a marker for platelet activation) [6] in CF. A similar defect of PGE_1 on platelet volume has been shown [11]. However, these phenomena seem not to be mediated via the Gs pathway, since platelet cAMP production, both basal and after stimulation with PGE_1 has been shown to be normal [12].

Only very recently, expression of CFTR (cystic fibrosis transmembrane conductance regulator) in human platelets has been demonstrated, which might be responsible for some of the intrinsic changes in platelet function seen in CF, although the exact mechanism for this platelet dysfunction still needs to be elucidated [13]. Nevertheless, O'Sullivan et al. showed an additional effect of CF plasma on non-CF platelet function [6]. When whole blood from healthy controls is diluted with CF plasma, there is an increased expression of P-selectin on platelets and likewise if CF blood is diluted with control plasma, there is a decrease in P-selectin expression [6]. This shows that the altered platelet function in CF is not only due to

the absence/reduction of CFTR function in platelets, but also to a hitherto unidentified plasma-borne factor.

As various studies have demonstrated [14;15], CF is characterized by an abnormal essential fatty acid state, especially with decreased docosa-hexaenoic acid levels (DHA, C22:6w3). The exact etiology of these abnormalities remains to be elucidated [14]. However, correcting this deficiency might ameliorate the course of the disease. For instance, supplementing DHA in an animal model of CF has shown promising effects [16;17] and several trials in patients studying the effect of DHA supplements on lung function have been undertaken (be it with variable results) [15]. The effect of DHA deficiency on platelet function remains unstudied.

Interestingly, essential fatty acids (omega-3 fatty acids) are known to modulate platelet activation. Therefore, they are currently studied in many trials for their effect on cardiovascular events and are endorsed by the American Heart Association in patients with documented coronary heart disease [18]. In vitro, DHA interferes with thromboxane A2 formation [19;20], is capable of inhibiting the thromboxane receptor [21] and could attenuate collagen-induced aggregation [19;20]. However, in randomized controlled trials in healthy controls an effect on platelet pro-aggregatory function could not be demonstrated [22]. However, the effect of eicosanoid-induced inhibition of platelet function (anti-aggregatory) still remains to be studied.

In this study, we wanted to investigate if a decrease in essential fatty acids could contribute to the increased platelet reactivity in CF. Therefore, we decided to characterize platelet function and inhibition thereof in CF, with emphasis on the cAMP mediated pathway, and study the effect of DHA supplementation on inhibition of platelet function.

Patients and methods

Platelet studies in patients with Cystic fibrosis patients and healthy controls

CF patients followed at the CF outpatient clinic at the University Hospitals Leuven, (Leuven, Belgium), in their usual state of health, were included in the **CF group**. The diagnosis of CF had been made according to international recommendations [23]. In the different platelet function assays we investigated platelets from 36 patients. Healthy volunteers were recruited among hospital and laboratory staff (**Control group**).

The study was approved by the KULeuven medical ethical committee and (patient or parental and control) informed consent was obtained for all patients and controls. Blood was drawn using a 21 gauge needle and Vacutainer tubes (Becton-Dickinson, Erembodegem, Belgium). The first tube was discarded to minimize artefactual platelet activation. All participants denied taking any non-steroidal anti-inflammatory drugs during the ten days preceding sampling.

Platelet counts and volumes (mean platelet volume) were determined on an automated hematology analyser, Sysmex XE-5000, Sysmex Europe GmbH (Norderstedt, Germany) and compared to institution-determined normal values for prepubertal children (<10y) and adults (>18y).

Platelet aggregation studies were done as described previously [24]. Briefly, blood was anticoagulated with 3.8% (wt/vol) trisodium citrate (9:1), and platelet-rich plasma (PRP), obtained by centrifugation (150 g for 15 minutes), was recentrifuged (3,000 g for 15 minutes) to produce platelet-poor plasma (PPP). Finally, the platelet count in the PRP was adjusted to 250×10^9 platelets per liter with PPP. Platelet aggregation was performed on dual-channel Chrono-Log aggregometers (Chrono-Log Corp., Havertown, Pennsylvania, USA) by simultaneous recording of four tracings. Aggregation studies were performed using Horm collagen (0.25 , 0.5 and 2 µg/ml; Nycomed Arzneimittel, Munich, Germany) or epinephrine (0.25, 0.5, 1µM (Sigma-Aldrich, Saint-Louis, MO, USA)) as an agonist. Aggregation-inhibition studies involved dose-response curves with Gs agonists prostacyclin (PGI_2, iloprost, Ilomédine; 0-5 ng/ml; Schering AG, Berlin, Germany), PGE_1 (Prostin; 0-1000 ng/ml; Pharmacia- Upjohn), which was added to PRP 1 minute before induction of aggregation by Horm collagen (2 µg/ml). The 50% inhibitory concentration (IC50) value was calculated by linear interpolation (Microsoft Office Excel 2003). The IC50 values ± SD for CF patients were compared with those obtained for a control subject using the same Gs agonist and

studied in parallel. In a separate experiment using control PRP, to study the influence of DHA on platelet aggregation-inhibition cis-4,7,10,13,16,19-Docosahexaenoic acid (Sigma-Aldrich) was added in vitro (final concentration of 50µg/ml). To accomplish this, DHA as a neat oil, was dissolved 1/200 in dimethylsulfoxide (DMSO). This was added to the PPP used for preparing the PRP (1% final concentration). PRP was reconstituted immediately before the aggregation. In the conditions without DHA, DMSO (vehicle) was added. In the reference PPP sample for the aggregation, DHA or DMSO was added in the same concentration.

To study **ATP secretion**, platelet aggregation and secretion were recorded in real time at 37°C with stirring after stimulation with Horm collagen (2 µg/ml). ATP secretion was determined by measuring the release of ATP using luciferin/luciferase reagent (Kordia, Leiden, Netherlands).

Platelet function was also assessed in the **Platelet Function Analyser-100** (Dade-Behring, Marburg, Germany). Blood was anticoagulated with 3.8% (wt/vol) trisodium citrate (9:1). The PFA-100 test measures the time to occlusion of a central aperture membrane coated with collagen/epinephrin. This closure time is a measure of shear-induced aggregation.

cAMP detection in platelets was performed as described previously [24]. Briefly, citrated PRP at $250x10^9$ platelets/l was obtained as described above and incubated with the Gs agonist PGE_1; 100 ng/ml (Pharmacia- Upjohn). In different samples, we arrested the reaction at different time points (0, 60, 120 and 300s) by adding 12% trichloroacetic acid and measured platelet cAMP using a cAMP enzyme-immunoassay (GE Heathcare, Amersham, Pharmacia Biotech).

Immunoblot analysis and **Vasodilator-stimulated phosphoprotein (VASP) phosphorylation**, a particularly well expressed target of cAMP activated protein kinase A in platelets [25], was performed as described previously [24]. Three hundred microlitres of citrated PRP at $250x10^9$ platelets/l was stimulated with PGE_1 (100 ng/ml;) for 1 or 5 min. A negative control sample without addition of PGE_1 was also prepared. Samples were immediately centrifuged at 16.000g for 30 sec at room temperature and the pellet was resuspended in 100 µL of sampling buffer (10 mM Tris pH 8.0, 1 mM EDTA, 5% SDS, 5% β-mercapto-ethanol, 2 mM NaF, 2 mM NaVO3, Bromphenol blue, one protease inhibitor cocktail tablet/50 ml). 5 µL proteins were separated on an SDS/PAGE acrylamide gel and transferred to Hybond ECL-nitro-cellulose membrane (Amersham, Pharmacia Biotech). The blots were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20

supplemented with 5% non-fat dry milk. Incubations overnight (4°C) with anti-VASP239 antibody from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) as primary antibody. and with HRP-conjugated secondary antibody (Rabbit anti-goat HRP-conjugated (Dako, Glostrup, Denmark), 2 h at room temperature) were also performed in Tris-buffered saline with Tween-20 supplemented with 5% non-fat dry milk. Staining was performed with the western blotting ECL detection reagent (Amersham Biosciences). Using the same methodology, the negative control sample was used for the detection of the catalytic subunit of protein kinase A with the **PKA catalytic subunit** (Santa Cruz) with as secondary a HRP-conjugated goat-anti-rabbit antibody (Dako). Expression was quantified by measuring the density of the bands (corrected for background), using the Image J software (National Institutes of Health).

Levels of docosahexaenoic acid in CF patients and controls were determined as described previously [26]. Briefly, blood samples (10 mL, SST-tube) were centrifuged at 1000g for 15 min at 4 °C. The serum was collected and stored at –80 °C for subsequent analysis involving acetylation and extraction. Fatty acid methyl esters were separated by use of a gas chromatograph (HP6890; Hewlett-Packard-Agilent) and identified and quantified by mass spectrometry (MS 5973; Agilent Technologies).

DHA supplementation experiment

Six healthy controls (3 males, 3 females) were selected to participate in a DHA suppletion experiment. The research protocol was approved by our local ethics committee, and all participants signed an informed consent. Alcohol intake was forbidden the two days preceding a blood sample. Furthermore, participants were instructed to continue their usual diet. Medication intake from a ten day period before the study until completion was prohibited.

At the beginning of the study all participants underwent a (fasted) vein puncture to determine baseline: full blood count performed on a cell-counter with human specific parameters (Cell Dyn 2000, Abbott, Chicago, IL, USA), determination of DHA levels, aggregation test to collagen 0.25 µg/ml, aggregation inhibition test with PGE_1 and PGI_2, VASP phosphorylation, cAMP determination (the latter two basal and after stimulation with PGE_1, see above). The shape change was also recorded.

Then, during ten days two capsules of 500 mg algal triacylglycerol oil with 40% of DHA (22:6n-3 were supplement three times daily (approximately 1.2mg of DHA) to the normal diet

(DHASCO oil, Martek Bioscience Corp., USA). After this supplementation period, a new (fasted) blood sample was taken to repeat the same baseline measurements.

Statistical analysis

SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results from larger groups (>25 subjects) are expressed as means ± standard deviation, the remainder as median / interquartile range. For differences between two continuous variables in two groups, the Mann-Whitney U test was used. For comparison of related samples, the Wilcoxon signed rank test was used. For comparison of categorical variables, Fischer's exact test (two-sided) was used. Boxplots for selected outcomes were constructed with a transverse line at the 50^{th} percentile, boxes extending from the 25^{th} to the 50^{th} percentile and whiskers extending from the 5^{th} to the 95^{th} percentile. A p-value <0.05 was considered statistically significant and a p-value <0.10, was considered as a statistical trend.

Results

Platelet characteristics

Platelet counts and volumes of F508del CF patients, in their usual state of health, were compared with healthy controls of similar age. In the group < 10 years of age there was no difference in platelet count (CF: 309 ± 65 * 10^9/L, n= 29; Control: 334 ± 79 * 10^9/L, n=31, p=0.270) and in mean platelet volume (CF: 9.74 ± 1.06 fL, n=29; Control: 9.33 ± 0.72 fL, n=31, p= 0.1). In the adult group (>18y), there was a significantly higher platelet count in the CF group (CF:301±89 * 10^9/L, n=52; Control: 260±52 * 10^9/L, n=50, p=0.003) There was no difference in mean platelet volume (CF: 11.57±3.83 fL, n=52; Control: 10.85±0.95 fL, n=50, p= 0.328).

Platelet function

PFA-100 analysis (measure of shear-induced platelet activation) demonstrates normal platelet function with a normal closure time in CF (Table 2.1). Aggregation in response to collagen (0.25, 0.5, 2µg/ml) and epinephrine (0.25, 0.5, 1µM) were normal in CF versus control (Table 2.1, Figure 2.1). Platelet ATP secretion in CF patients upon stimulation with collagen 2µg/ml was also normal compared to controls (Table 2.1). (all p>0.05)

Figure 2.1 : Platelet stimulation in CF and controls

Boxplots of maximal amplitudes of platelet aggregations in CF patients (shaded boxes) and healthy controls (open boxes).

Each dot represents a single measurement. Platelets were stimulated with collagen: 0.25, 0.5 and 2µg/ml (**top**) or with epinephrine 0.25, 0.5 or 1µM (**bottom**). p=NS

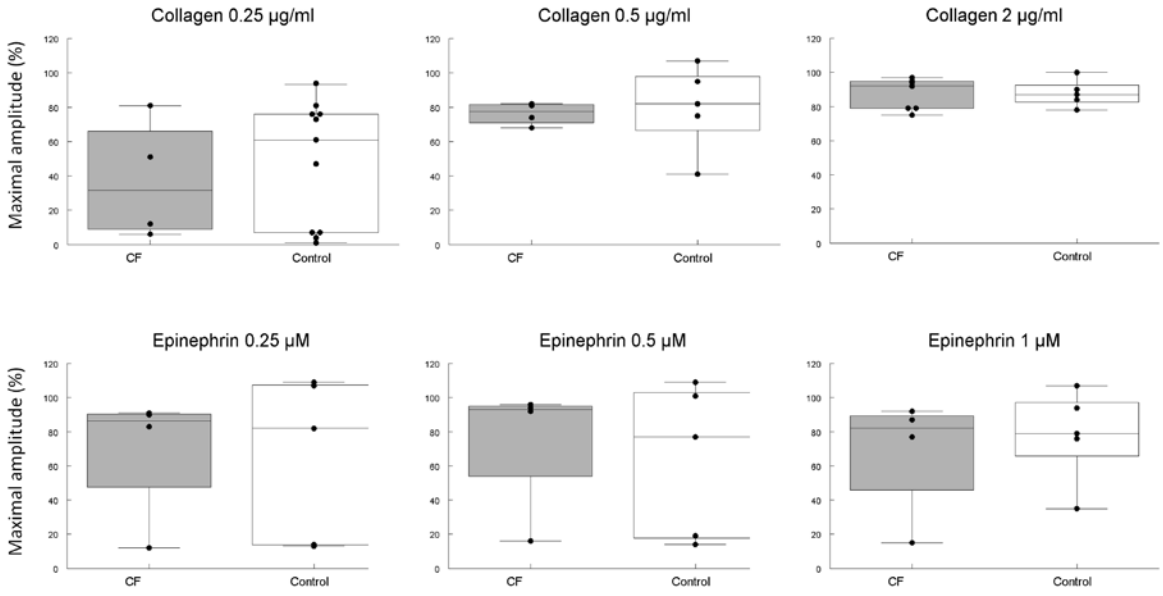
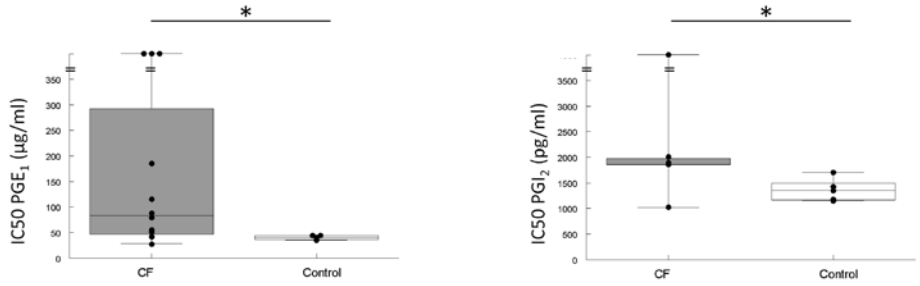
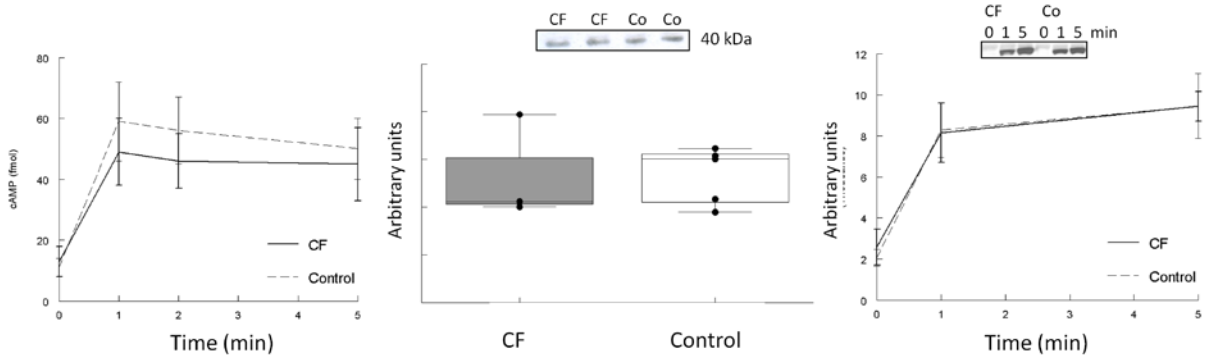


Figure 2.2 : Inhibition of platelet stimulation in CF and controls
Left: Boxplots of inhibitory concentration (IC)50_{PGE1} in CF (n=12, shaded boxes) vs controls (n=5, open boxes) and **Right** $IC50_{PGI2}$ in CF(n=7, shaded boxes) versus controls(n= 5 open boxes). Each dot represents an individual measurement. * p<0.05



Left: cAMP production after stimulation with PGE_1 100 µg/ml. Means and standard deviations are shown of CF patients (full lines, n=8) and controls (broken lines, n=8).
Middle: Expression the catalytic subunit as determined by western blot (insert), with the quantification in CF patients (shaded boxes, n=4)) versus controls (open boxes, n=5)
Right: VASP phosphorylation: basal and after stimulation with PGE_1 100 µg/ml, as determined by western blot (insert) at t= 0, 1 and 5 minutes, with quantification in CF patients (shaded boxes, n=4) versus controls (open boxes, n=6). Means and standard deviations are shown. p=NS



The aggregation-inhibition Gs test for which the collagen-induced aggregation is inhibited with PGE_1 or PGI_2 showed a marked decreased inhibition in the CF group compared to controls (increased inhibitory concentration (IC)50_{PGE1} 83.80 / 189.28 (n=12) vs 40.60 / 8.07 µg/ml,(n=5) p=0.027, $IC50_{PGI2}$ 1.86 / 0.15 ng/ml (n=7) versus 1.34 / 0.40 (n= 5) p=0.048) (Figure 2.2, top panel).

To further study the PGE_1 stimulated Gs pathway, we determined intracellular cAMP levels. Basal concentrations of cAMP and stimulation thereof with PGE_1 (100 µg/ml) showed equal levels and changes in the CF group versus controls. (p>0.05 at all time points, Figure 2.2, lower panel).

Table 2.1: Platelet pro-aggregatory function in CF patients and controls.

		Control (Median/IQR (n))	CF (Median/IQR (n))	p-value
closure time PFA-100 (s) (collagen/epinephrine cartridges)		97 / 13 (7)	92,5 / 22,75 (10)	0.417
Amplitude aggregation (%) with collagen	2µg/ml	87 / 14 (5)	92,5 / 20,5 (12)	0.799
	0.5µg/ml	82 / 43 (5)	77,5 / 12,25 (4)	0.413
	0.25µg/ml	7 / 82,5 (5)	31,5 / 66 (4)	0.730
Amplitude aggregation with epinephrine	1µM	79 / 45 (5)	82 / 60,25 (4)	0.730
	0.5µM	77 / 88,5 (5)	93 / 60,5 (4)	1.000
	0.25µM	82 / 94,5 (5)	86,5 / 61 (4)	0.905
ATP secretion after stimulation with collagen 2µg/ml (µM)		1.86 / 1.005 (40)	1.16 / 2.225 (8)	0.107

To study PKA levels (a cAMP-dependent kinase) we performed immunoblot analysis. The catalytic subunit of the PKA was equally expressed in CF patients as in controls (as determined by western blot, Figure 2.2, lower panel).

To further study the PKA-activity, we determined the phosphorylation status of its major target, VASP. The VASP phosphorylation under basal conditions and after stimulation with PGE_1 however, showed no differences between CF patients and controls (Figure 2.2, lower panel).

DHA state in DF508 CF patients

DHA levels were determined in 115 CF patients (all F508del, in their usual state of health, 63 male, 52 female, age 15.88±9.46y) and in 58 controls (22 male, 31 female, age 19.87±12.95y). There was no difference in age (p=0.157) or in gender (p=0.10) between patients and controls. DHA levels were significantly lower in CF patients compared to the healthy controls (30.97± 19.31µg/ml versus 46.47 ± 24.69 µg/ml, p<0.00001). (Figure 3.3)

DHA and platelet function in vitro

To test the influence of DHA on inhibition of the platelet aggregation in vitro, we decided to add 50µg/ml (i.e. approximating the normal serum concentration, see above) to the aggregation induced by collagen (2µg/ml) and to the aggregation-inhibition Gs test using control platelets (Figure 2.4).

Figure 2.3: DHA level (ng/ml)
Boxplots of DHA levels in CF patients (shaded box) versus controls (open box): 30.97± 19.31µg/ml (n=115) versus 46.47 ± 24.69 µg/ml (n=58). Each dot represents an individual measurement. ** p<0.0001

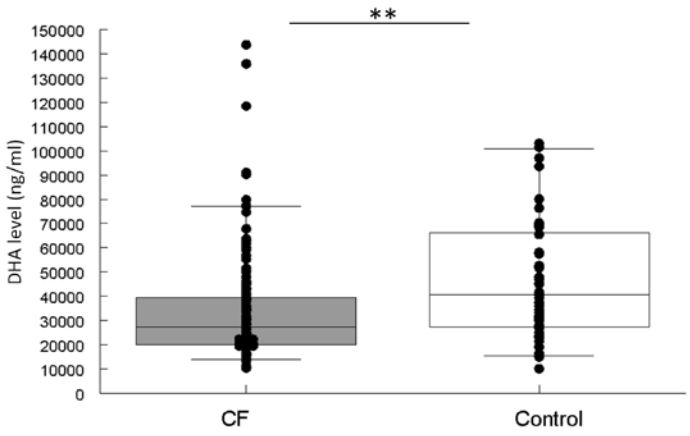
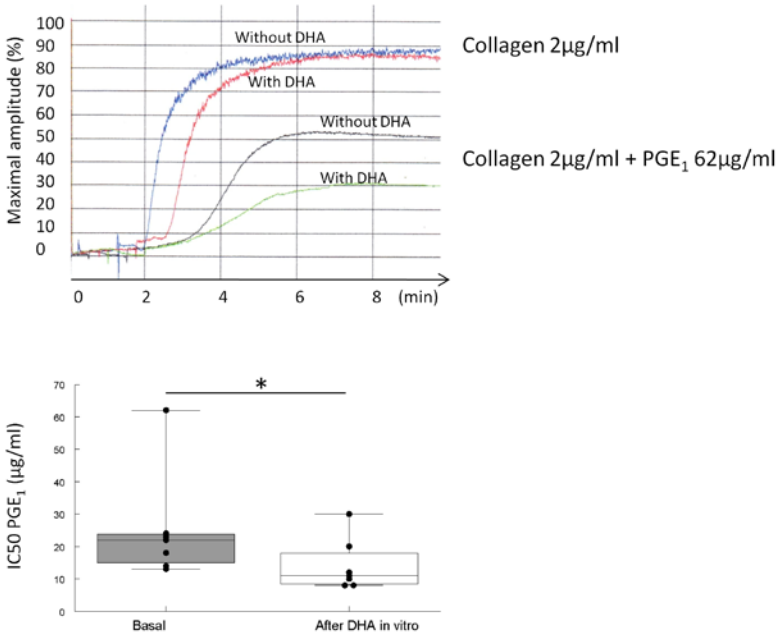


Figure 2.4: DHA and platelet inhibition in vitro
Top: Tracing of an aggregation with collagen either with or without the preincubation with PGE_1 in the presence or absence of DHA.
Bottom: Boxplots of inhibitory concentration $IC50_{PGE1}$ without DHA (shaded boxes) versus with DHA (open boxes), n=7, Each dot represents an individual measurement.
*p< 0.05.



Addition of DHA did not affect the collagen induced aggregation (without DHA (with DMSO): 89 / 23% versus with DHA: 81 / 15%; n= 7; Wilcoxon signed rank: p=0.176).

However, addition of DHA resulted in a significant increased inhibition of collagen aggregation using PGE_1 (or a decrease in $IC50_{PGE1}$ without DHA: 22.92 / 10.26µg/ml versus with DHA 11.14 / 12.81, n=7, Wilcoxon signed rank test: p= 0.043).

DHA supplementation in healthy controls

Treatment with DHA was well-tolerated. Platelet functions and measurements were performed before and after a 10 day supplementation of DHA. This leads to an increase of plasma DHA level (currently only determined in 4 subjects) from 38.54 / 24.83 µg/ml to 90.43 / 21.80 µg/ml (or 197% of the initial value). There was good compliance, as evidenced by the achieved blood levels.

There was no change in platelet count (287 / 58*10^9/L before, 261 / 43*10^9/L after, p=0.345) or in MPV (7.76 / 0.97fL before, 7.71 / 0.59 fL after, p=0.917).

The aggregation had a slight trend towards a decrease in aggregation response after stimulation with high dose of collagen: 2µg/ml (83.00/3.75% before to 78.5/8.25 after, p= 0.066) but no difference was seen after stimulation with low dose of collagen: 0.25µg/ml (67.00/24.75% before, 66.00/7.5% after, p=0.917). (Figure 2.5, top panel)

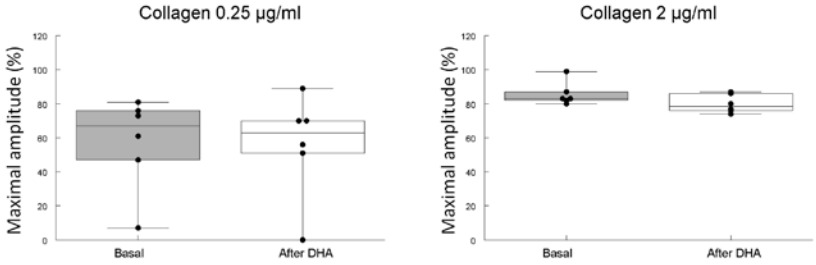
Supplementation of DHA did not influence depth of platelet shape change after stimulation with collagen 2µg/ml (5.80/2.50% to 8.55/1.30%, p=0.344).

Supplementation of DHA led to a trend in increase of inhibition by PGE_1 of the aggregation with collagen 2µg/ml (i.e. decrease in $IC50_{PGE1}$ 138.13 / 157.88 before to 69.16 / 48.28 µg/ml after, p=0.075) and to a significant increase in inhibition by PGI_2 ($IC50_{PGI2}$ 1.857/0.781 before to 1.212/0.421 ng/ml after, p=0.028, Figure 2.5, middle panel).

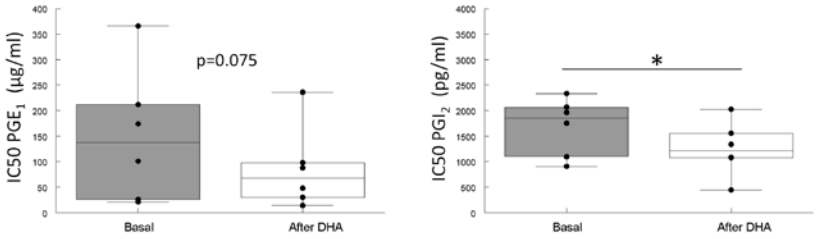
Basal concentration of the major inhibitory second messenger cAMP and stimulation thereof with PGE_1 (100µg/ml) showed equal levels and changes before and after DHA supplementation (p at all time points >0.05, Figure 2.5, bottom panel).

The VASP phosphorylation was again not different before and after supplementation with DHA (Figure 2.5, bottom panel).

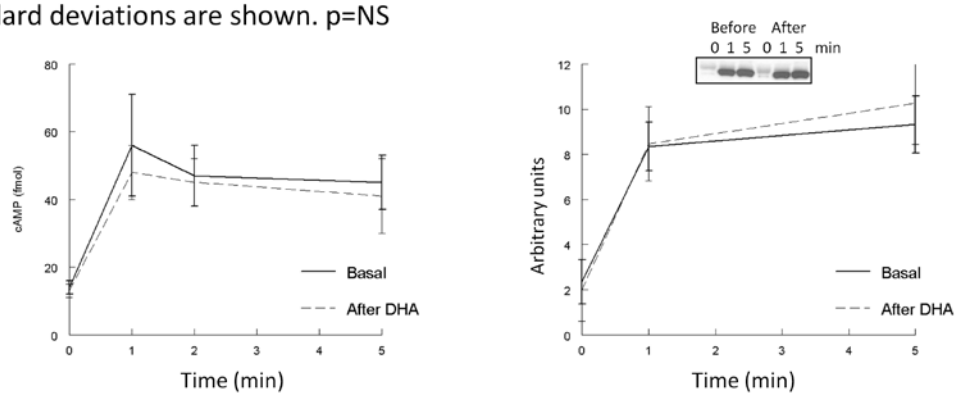
Figure 2.5: DHA level in vivo supplementation and platelet function
Stimulation:
Boxplots of maximal amplitudes of platelet aggregations in before administration of DHA (shaded boxes) and after (open boxes). Each dot represents a single measurement. Platelets were stimulated with collagen: 0.25 **(left)** and 2µg/ml **(right)**. p=NS



Inhibtion
Left: Boxplot of $IC50_{PGE1}$before DHA supplementation (shaded box) versus after DHA supplementation (open box) and **Right:** $IC50_{PGI2}$ before (shaded box) and after (open box) DHA supplementation. Each dot represents an individual measurement.



Left: cAMP production after stimulation with PGE_1 100 µg/ml. Means and standard deviations are shown from before DHA supplementation (full lines) and after DHA supplementation (broken lines). p=NS
Right: VASP phosphorylation: basal and after stimulation with PGE_1 100 µg/ml, as determined by western blot (insert) at t= 0, 1 and 5 minutes, with quantification from before DHA supplementation (shaded boxes) versus after DHA supplementation (open boxes). Means and standard deviations are shown. p=NS



Discussion

In the present work we showed a normal platelet pro-aggregatory function but a defective inhibition thereof by PGE_1 and PGI_2 in CF patients. The observed Gs hypofunction is however not mediated by cAMP. Decreased levels of DHA can be an explanation for this, as DHA supplementation in healthy volunteers leads to a gain in inhibition, likewise, not mediated by cAMP.

We found that platelets from CF patients have a normal mean platelet volume. However, there is a relative thrombocytosis in older CF patients. This was already previously shown and could be attributed to inflammation (reactive thrombocytosis) [7].

On a functional level, we showed platelet aggregation is actually normal in CF patients. We could not repeat previous findings of impaired platelet hyperaggregability. Reported literature on this aspect stems from more than 20 years ago, so methodology and clinical characteristics of patients with CF have evolved [7;8]. Our findings are in accordance with other authors showing normal platelet aggregation in CF [4;9]. It is also important to note that we used platelet preparations from patients attending the outpatient CF clinic that were in their usual state of health, so without acute infectious exacerbations. These findings suggest stimulatory pathways are intact. Moreover, we are the first to show that shear-induced platelet activation (closer to physiologic circumstances) is normal, as is platelet secretion following their activation (as determined by ATP release).

Interestingly, we did find a Gs hypofunction in CF patients as the inhibition of their platelet function by PGE_1 or PGI_2 is reduced. The only known pathway in platelets leading to this inhibition is via the Gs signaling pathway (activation of adenylate cyclase, subsequent production of cAMP and thereby activation of PKA with the phosphorylation of different targets leading to the inhibition of platelet aggregation). However, thorough investigation of this pathway, with determination of cAMP levels, PKA quantity and PKA activity (phosphorylation of VASP as a major PKA target), could not show any abnormalities. This is in keeping with published literature [6;11;12]. We are the first to show that this response is equally impaired to PGI_2, an endothelium-produced physiologic factor, contrary to PGE_1, a pharmacologic dilator [25].

This has relevance in vivo. Under physiologic circumstances platelet activation and inhibition are kept in tight equilibrium. Alteration of the inhibition can cause a shift towards either

bleeding (gain of inhibition) or thrombosis (loss of inhibition), as evidenced by various diseases [5].

It is interesting to note that in CF patients there is a particularly high incidence of deep vein thrombosis associated with peripherally inserted central catheters [27]. In fact the incidence of 3.7% in CF patients is at the high end of the expected incidence in non-CF, non-malignant populations [27]. CF constitutes also a risk factor for recurrent venous thrombosis in children [28]. In this study, none of these patients had historical or laboratory evidence of inherited thrombophilia and the inflammation induced by *Burkholderia cepacia* was considered a contributory factor. Whether less inhibited platelets play a role in this remains to be determined.

As the exact mechanisms for these alterations remain elusive, and a hitherto unrecognized plasma-borne factor appears to play a role [6], we surmised DHA could play a role. Indeed, we showed DHA levels are decreased in cystic fibrosis (in keeping with various other publications [14;15]). Interestingly, we found that adding DHA, at a physiologically relevant concentration, in vitro, could increase the inhibitory effect of PGE_1 on collagen-induced platelet activation. Likewise, we could demonstrate, by means of a supplementation trial, that this effect is achievable in vivo. We are the first to demonstrate that DHA supplementation leads to an increase of the inhibitory effect of endothelium-produced anti-aggregatory eicosanoids. As in CF, this seems not to be mediated via the Gs pathway (as evidenced by normal cAMP concentrations and normal VASP phosphorylation (both basal and after PGE_1 stimulation)).

Although we did not yet show that supplementation of the DHA deficiency in CF could normalize the decreased inhibition, we did show that adding DHA to healthy controls increases inhibition. Thereby it seems unlikely this would not be the case in CF. Moreover, there is an overlap in DHA concentrations between controls and CF patients and our supplementation in controls with lower DHA levels would be similar as in the CF patients. Nevertheless, this could easily be confirmed in a larger DHA supplementation trial in CF as one of the outcomes.

The exact mechanisms by which DHA affects inhibition of platelet function is an exciting field for further research. In numerous cells there are reports on cAMP-independent Gs protein mediated pathways, for instance in smooth muscle cells [29] and platelets [30]. The

exact mechanisms remain unknown, but the Gs protein beta-gamma complex could play a role in this.

Hereby, our findings could not replicate platelet hyperaggregability per se, but we did see convincing evidence of a decreased inhibition. Thereby platelets could indeed play a role in the physiopathology of CF disease. As already stated, platelets are thought to play a role in the development of CF lung disease [1;2].

These findings could also be relevant to other infectious and inflammatory diseases. For instance in inflammatory bowel disease, where platelets play a similar role in the pathogenesis [3;31]. As in CF, there is increased P-selectin expression and there are increased plasma markers of platelet activation [3]. The inhibition of platelet aggregation remains to be studied. Interestingly, fish oil substitution (leading to an increase in DHA and eicosapentaenoic acid) could reduce the rate of relapse in inflammatory bowel disease [31].

These findings are not only relevant to infectious/inflammatory diseases as CF and inflammatory bowel disease. Humans are rather poor synthesizers of DHA and due to the evolutionary change in our diet there is a subclinical DHA deficiency in the entire population [32]. This is epidemiologically related to the rise in cardiovascular, inflammatory and psychiatric disorders [32].

Regarding cardiovascular disease, DHA supplementation leads to less atherogenic larger LDL particles [22]. However, in 8/17 randomized controlled trials, there is rather an increase in LDL levels [22]. Of course, platelets are important mediators in cardiovascular events. While previous studies in healthy controls could not demonstrate an effect on platelet function [22], we have shown here that DHA supplementation leads to increased inhibition in platelets, which is probably equally important in vivo.

Conclusion

We have demonstrated normal pro-aggregatory function of CF platelets by conventional testing. However, there is a decreased platelet inhibition of collagen-induced aggregation with either PGE_1 or PGI_2. This is not mediated by the traditional Gs pathway (cAMP, PKA). We have shown that DHA, a plasmatic factor deficient in CF, can mediate a Gs independent increase in platelet inhibition. Supplementation in healthy controls showed to be effective in modulating this inhibition, supplementation in CF patients remains to be studied.

This finding reinforces the importance of essential fatty acid disturbances, and might play a role in the suspected beneficial effects of DHA supplementation.

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Chapter 3: Lung transplantation in cystic fibrosis normalizes essential fatty acid profiles

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Abstract

Rationale

Cystic fibrosis is a devastating disease. Disorders in essential fatty acid state are increasingly reported and various supplementation trials have been performed in an attempt to improve outcomes. However, the mechanisms leading to these disturbances remain elusive.

Objective

We wanted to investigate the role of the diseased cystic fibrosis lung on fatty acid profiles.

Methods and Measurements

We compared fatty acid profiles in cystic fibrosis patients that underwent a lung transplantation (n=11) to age-matched healthy controls and homozygous F508del patients (n=22 each).

Main Results

Compared to healthy controls, in cystic fibrosis patients, there were decreased levels of docosahexaenoic, linoleic and arachidonic acid and increased levels of mead acid. In patients that had undergone a lung transplantation, there were normal levels of docosahexaenoic, linoleic and arachidonic acid. Mead acid did not decrease significantly.

Conclusions

The diseased CFTR deficient lung is a major determinant in the disturbed fatty acid profile in cystic fibrosis.

Introduction

Cystic fibrosis (CF) is the most frequent lethal genetic disorder within the Caucasian race, with an incidence of 1 to 2500-400. Due to the current medical treatment, the median survival of these patients rose to more than 35 years. Pulmonary problems are the main cause of death. In an effort to further improve care, more and more attention is paid to metabolic disturbances, as for instance diabetes and also essential fatty acid (EFA) deficiency [1].

Abnormal FA profile has been reported in children with cystic fibrosis since many years [2]. Renewed interest in this subject emerges from the ability to influence FA profiles by supplements, thereby adding to the dietary strategies to obtain an optimal nutritional state [1]. Moreover, disturbances in fatty acid profiles have been implicated in the predisposition to lung disease [3]. Also, in a CF mouse model supplementation of one of the FA (docosahexaenoic acid (DHA, C22:6 n-3)) was beneficial to the *Pseudmonas* endotoxin-enhanced lung inflammation, CF pancreatic, ileal and liver disease [4;5]. Nevertheless, randomised controlled trials are still needed to confirm or refute beneficial effects of EFA supplementation in CF patients [1].

The major EFA defect in CF patients seems to be an increased release of arachidonic acid (AA, C20:4 n-6), the most important metabolic product of linoleic acid (LA) [6]. In serum as well as in nasal and rectal biopsy samples, a high ratio of AA to DHA is seen [7]. In addition there is a decrease in plasma levels of inoleic acid (LA, C18:2 n-6) and DHA and a compensatory increase in levels of eicosatrienoic acid (C20:3 n-9) (also known as mead acid (MA)) [1;8-10].

The aetiology of this abnormal fatty acid profile remains obscure. Initially this was thought to reflect fat malabsorption linked to the exocrine pancreatic insufficiency. However, it even occurs in well-nourished young cystic fibrosis patients [11] which makes this less probable. It has been suggested that there is a disturbance in essential fatty acid metabolism related to the CF-genotype [12]. This is supported by the observations that minor changes in the essential fatty acid patterns are even seen in CF-heterozygotes [7;13].

Undisputedly, lung disease is the most important aspect of cystic fibrosis. There have been no trials (including a placebo group) showing improvement or stabilisation of lung function after improvement of fatty acid state. Although lung function (measurement of the forced expiratory volume in one second, FEV_1) has been related to CF fatty acid abnormalities [14]

and as already stated, implicated in the predisposition of CF lung disease [3], its precise relation is not clear. Moreover, interdependency of clinical variables (e.g. genotype, pancreatic state and lung function) poses a problem to independently evaluate its relationship. Therefore, we decided to study the reverse: the influence of a diseased CF lung on the fatty acid state in CF patients We compared the fatty acid state in CF patients and with and without lung transplantation.

Patients and methods

Patients

Plasma levels of fatty acids were determined in a group of 11 DF508/DF508 CF patients who underwent an isolated lung transplantation at least one year before the onset of the study **(CF-transplant group)**. All patients were followed at the CF clinic of the university hospital Gathuisberg, (Leuven, Belgium). 10 patients underwent a double lung transplantation, one patient a single lung transplantation. This group was age-matched with two DF508/DF508 CF patients drawn from our cohort of 225 CF patients (in an age-sorted patient list the preceding and the following patient were selected, **CF group)**. Additionally we collected two healthy control samples per CF lung transplant (again one older and one younger) **(Control group)**. The plasma fatty acid levels of these three groups were compared.

At the time of the plasma fatty acid measurements, or as close to it as possible, the following clinical information was retrieved from the patients clinical files: age; age at diagnosis; gender; history of meconium ileus; lung function tests (FVC, FEV1 expressed as % predicted for sex, height and age according to Knudson, within 3 months of the EFA measurement); chronic *Pseudomonas aeruginosa* colonization (present/absent: repeated isolation of *Pseudomonas aeruginosa* (at least 3 positive sputum samples, with at least 1 month interval over at least a 6 month period [15]); weight, height, BMI and their respective z-scores (using recent Flemish reference values); serum levels of vitamin E and cholesterol, dietary intake: daily average for total caloric intake and fat intake calculated from prospective 6 day written diary and fish intake during the last 2 months as number of fish meals/week. Pancreatic insufficiency was defined by the need for pancreatic enzyme supplement for the treatment of clinically overt steatorrhoea.

This study was approved by the local ethics committee and (patient and/ or parental) informed consent was obtained for all patients.

Plasma fatty acid determination

FA levels of the patients and controls were determined as previously reported [16]. The possible differences between fed/fasted states and with respect to diurnal variation were mitigated by the presence of reports suggesting such variations being negligible for phospholipids [14]. Hence, blood samples were drawn at random times. Briefly, blood samples (5 mL on EDTA) were centrifuged at 1000g for 15 min at 4 °C. The plasma was

collected and stored at –80 °C for subsequent analysis. In glass tubes containing 100 µL plasma, 20 µL of the internal standard solution (providing 50 mg C19:0/L) and 2 mL of a methanol/toluene (4/1, vol/vol) mixture were added. Samples were vortex-mixed for 30 s, and we carefully added 200 µL acetyl chloride dropwise while swirling the tubes. The tubes were capped under nitrogen and transferred to a heating oven at 103 °C for 90 min. After cooling, the tubes were treated by addition of 5 mL 6% K_2CO_3 and of 3 mL hexane. Recapped tubes were vortex-mixed for 1 min and centrifuged for 7 min at 500g. The upper organic phase was collected and the lower phase was again extracted by adding 1 mL hexane. The two upper combined phases were washed with 1 mL distilled water, followed by a new centrifugation. The final organic phase was collected and evaporated under nitrogen to dryness at 40 °C. The dry residue was then redissolved in 200 µL hexane and transferred to capped vials for further analysis.

Fatty acid methyl esters were separated by use of a gas chromatograph (HP6890; Hewlett-Packard-Agilent, Santa Clara CA, USA) equipped with a capillary column (DB-FFAP), 30 m by 0.25 mm ID by 0.25 µm (J and W Scientific, Agilent Technologies) and identified and quantified by mass spectrometry (MS 5973; Agilent Technologies). The temperature program was as follows: initially 130 °C for 1 min; ramp: 4 °C/min to 178 °C, 1 °C/min to 210 °C, and 40 °C/min to 245 °C with a 7-min hold. Helium was used as carrier gas, under a flow rate of 2.5 mL/min. The fatty acid methyl esters were idnetified by their respective retention times and by comparison with spectra of pure reference substances. Intra- and interassay CVs were <3% and <5.7%, respectively for all analysed fatty acid methyl esthers (except for gamma-linolenate methyl ester, with 9.3%).

Statistical analysis

SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Demographic variables are expressed as mean ± standard deviatiation. As fatty acid levels were not normally distributed, they are expressed as median (interquartile range). For differences between two continuous variables in two groups the Mann-Whitney's U-test (with exact correction if groups were too small) was used. To compare categorical variables Fisher exact test (2-sided) was used. For correlations within a group the Spearman Rank Correlation coefficient was used. A p-value <0.05 was considered statistically significant.

Results

Demographic variables (see Table 3.1)

All CF patients were DF508 homozygous and pancreatic insufficient. There were no differences between the CF patients with and without a lung transplantation regarding age, sex, age at diagnosis, lung function, biometric variables (height, weight and BMI z-score), fish intake or history of meconium ileus. However transplanted patients had decreased caloric intake (also decreased fat intake), higher levels of serum cholesterol and vitamin E. All had been chronically colonized with *Pseudomonas auruginosa* (contrary to the non-transplanted group (14/22, see Table 3.1). Patients that underwent a lung-transplantation did so 4.32+/- 2.08 year before the plasma FA determination.

Table 3.1: Baseline characteristics.

		CF transplant group (n=11)	CF group (n=22)	P-value (TXvsCF)
Gender M/F		4/7	13/9	0.28
Age(y)		27.5 +/- 7.8	25.9 +/- 6.8	0.42
Age at diagnosis (y)		2.33 +/- 2.70	3.31 +/- 7.82	0.07
Age at transplantation (y)		23.16 +/- 7.05	Not performed	-
Lung function	FVC1 (%pred)	80 +/- 13	79 +/- 19	0.96
	FEV1 (%pred)	74 +/- 20	60 +/- 23	0.11
Chronic *Ps aer*	(n)	11 (pretransplant)	14	**0.031**
Biometrics	Length (z-score)	-1.22 +/- 0.93	-0.87 +/- 1.00	0.32
	Weight (z-score)	-1.15 +/- 1.00	-1.02 +/- 1.27	0.81
	BMI (z-score)	-0.52 +/- 0.70	-0.56 +/- 1.07	0.72
Dietary intake	Kcal/d	2.382 +/- 360	2.985 +/- 465	**<0.001**
	Fat (Kcal/d)	890 +/- 205	1.196 +/- 206	**<0.001**
	Fish intake (times/week)	0.95 +/- 0.61	0.77 +/- 0.67	0.51
History of meconium ileus	(n)	1	6	0.38
Serum cholesterol	mg/dL	155.73 +/- 44.00	125.18 +/- 35.52	**0.032**
Serum vitamin E	mg/dL	11.99 +/- 4.52	7.57 +/- 2.94	**0.003**

Analysis of fatty acids (see Table 3.2)

Table 3.2: Fatty acid state of controls, CF patients and CF lung transplanted patients.

Fatty acids (μM)	Control	TX	CF	Control versus TX (p)	CF versus TX (p)	CO versus CF (p)
Lauric acid (c12:0)	6.92 (13.93)	14.58 (30.93)	3.93 (12.85)	0.26	0.23	0.59
Myristic acid (c14:0)	120.05 (103.98)	146.37 (311.63)	103.3 (106.58)	0.06	0.08	0.81
Hexadecanal dimethyl acetate (c16:0)	24.29 (10.14)	24.36 (15.64)	13.61 (12.01)	0.87	**0.003**	**<0.001**
Palmitic acid (c16:0)	1852.99 (1274.63)	2671.18 (2454.21)	1723.83 (832.58)	0.13	**0.048**	0.30
Stearic acid (c18:0)	617.19 (267.82)	618.76 (393.57)	488.98 (135.64)	0.90	0.06	**0.008**
Arachidic acid (c20:0)	18.56 (8.47)	16.29 (12.13)	10.38 (3.67)	0.38	**0.024**	**<0.001**
Behenic acid (c22:0)	49.72 (15.85)	45.01 (26.62)	28.82 (8.18)	0.09	**0.040**	**<0.001**
Lignoceric acid (c24:0)	39.27 (14.55)	34.08 (19.53)	20.1 (6.49)	**0.04**	**0.012**	**<0.001**
Myristoleic acid (c14:1)	2.58 (31.85)	2.00 (21.67)	2.38 (14.58)	0.81	0.90	0.65
Palmitoleic acid (c16:1)	153.22 (141.19)	316.58 (312.71)	277.24 (221.21)	0.17	0.96	**0.024**
Oleic acid (c18:1n-9)	1672.74 (1226.72)	2176.53 (2457.35)	1558.23 (661.81)	0.07	0.05	0.94
Vaccenic acid (c18:1n-7)	136.87 (65.91)	198.66 (179.77)	151.74 (95.87)	**0.011**	0.08	0.39
Eicosenoic acid (c20:1n-9)	6.1 0 (7.87)	10.66 (25.14)	6.96 (3.77)	0.13	0.18	0.82
Erucic acid (c22:1n-9)	0.00 (0.00)	0.00 (0.17)	0.00 (0.00)	0.84	0.99	0.52
Nervonic acid (c24:1n-9)	77.68 (39.79)	76.84 (28.16)	73.82 (19.93)	0.96	0.32	0.12
Alpha-Linolenic acid (c18:3n-3)	48.07 (49.29)	61.61 (71.12)	32.48 (28.5)	0.49	0.08	0.06
Eicosatrienoic acid n-3 (c20:3n-3)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.69	0.69	1.00
Eicosapentaenoid acid (c20:5n-3)	54 (40.52)	47.97 (89.29)	55.77 (32.78)	0.87	0.81	1.00
Docosatrienoic acid (c22:3n-3)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.00	1.00	1.00
Docosapentaenoic acid n-3 (c22:5n-3)	28.91 (16.95)	33.32 (38.6)	29.52 (25.54)	0.34	0.34	0.85
Docosahexaenoic acid (c22:6n-3)	135.67 (60.13)	165.66 (113.87)	89.22 (63.4)	0.34	**0.021**	**0.003**
Linoleic acid (c18:2n-6)	2398.51 (918.4)	2172.24 (930.35)	1478.86 (633.75)	0.61	**<0.001**	**<0.001**
Gamma-Linolenic acid (c18:3n-6)	26.19 (24.22)	28.38 (35.9)	27.18 (30.69)	0.72	0.82	0.34
Dihomo-gamma-Linolenic acid (c20:3n-6)	121.66 (95.53)	150.75 (109.13)	119.63 (70.38)	0.38	0.26	0.77
Arachidonic acid (c20:4n-6)	561.84 (322.29)	682.6 (276.12)	444.38 (185.39)	0.67	**0.015**	**0.021**
Docosatetraenoic acid (c22:4n-6)	11.01 (8.99)	12.02 (15.72)	11.04 (8.68)	0.13	0.23	0.59
Docosapentaenoic acid n-6 (c22:5n-6)	11.41 (7.95)	11.96 (32.86)	13.32 (8.37)	0.20	0.64	0.22
Mead acid/Eicosatrienoic acid n-9 (c20:3n-9)	8.41 (17.66)	23.56 (41.19)	29.91 (43.45)	**0.014**	0.32	**<0.001**
Total	8327.04 (4981.21)	10046.33 (9056.46)	7009.9 (3081.33)	0.26	0.02	0.06

Total plasma fatty acid concentration was significantly higher in lung transplanted patients compared to CF patients.

Saturated fatty acids: There were no significant differences between the CF patients who underwent a lung transplantation and the healthy controls, except for Lignoceric acid (c24:0). This was decreased, however, not to the extent as in the non-transplanted CF group. In the CF group, levels of various unsatured fatty acids were significantly decreased both compared to the control group as also compared to the CF transplant group. (see Table 3.2)

Mono-unsaturated fatty acids: There were few differences between the groups. Only Vaccenate (c18:1n-7) was higher in the CF transplant group compared to the control group (not to the CF group) and Palmitoleic acid (c16:1) was higher in the CF group compared to the control group (not to the CF transplant group) (see Table 3.2).

Poly-unsaturated fatty acids (PUFA): In the CF groups there was a significant decrease in DHA, LA, AA compared to both the control group and the CF transplant group. On the contrary these fatty acids were not different between the CF transplant group and the control group. MA was significantly increased in the CF group and also in the CF transplant group compared to the control group. MA was the only PUFA that was different between the control and the CF transplant group (see Figure 3.1)

Fatty acid profiles expressed as mol% relative to the total fatty acid content were also calculated. These results can be found in the supplementary table at the end of this chapter. With regard to the PUFA (mol%) we saw that DHA levels rose after lung transplantation to normal values and that MA decreased, but still remained significantly elevated above control values. Regarding the n-6 series LA and AA remained low after lung transplantation (Supplementary table).

Relation between PUFA and other clinical variables

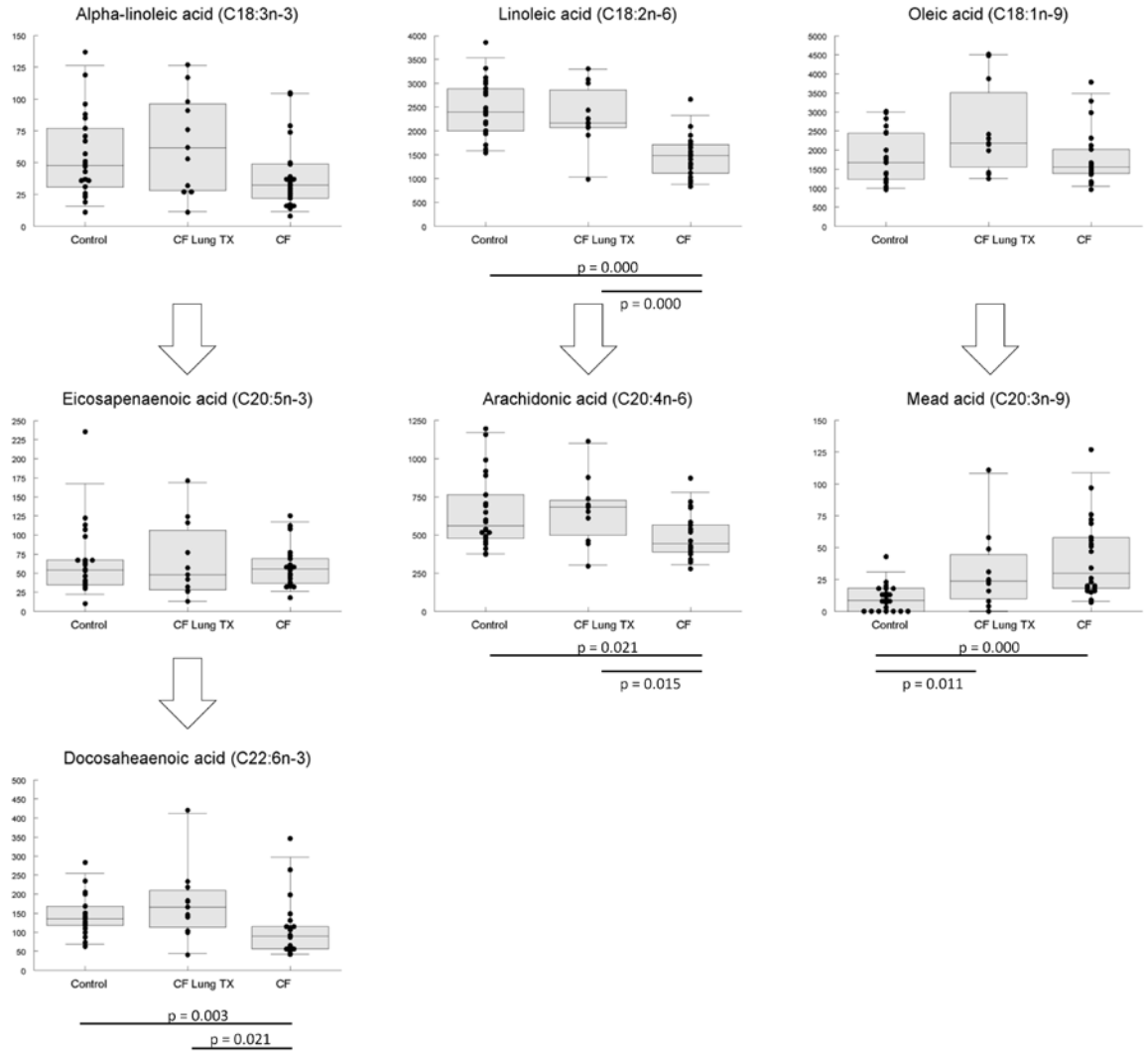
Further we studied the relation between the PUFA state (Table 3.2) and significantly changed clinical values.

Within the CF group there was no difference with regard to one of the four significantly changed PUFA (DHA, LA, AA, MA) and *Pseudomonas aeruginosa* colonization ($p \geq 0.05$ for all), caloric intake, fat intake or vitamin E levels ($p \geq 0.05$ for all). However cholesterol

levels were positively correlated with absolute levels (expressed as µM) of DHA (r=.531), LA(r=.528) and AA (r=.630).

Within the transplanted group there was the same significant correlation with the cholesterol (DHA r=.838, LA r=.752, AA r=.866), but also with the decreased fat intake (AA r= -.656 and MA r= -.715). Vitamin E (which minimizes DHA peroxidation [5]) also correlated positively with DHA level (r=.636) ($p<0.05$ for all correlations).

Figure 3.1 : Boxplots showing PUFA state in the three groups
Control group (left), CF group (middle), CF transplant group (right). Each dot represents an individual measurement.



Ratios between PUFAs (Table 3.3)

There was evidence for an essential fatty acid deficiency (documented by an elevated ratio of MA to AA [11;14]) in CF patients (above the 0.02 cut-off) and was more than in the CF transplant group. AA level multiplied with DHA have (suggested as diagnostic marker for CF [16]) was decreased in CF patients, but not in CF lung transplanted patients. The ratio of AA to DHA (a marker of inflammatory state and a reflection of the n-6 to n-3 composition [7]) was not significantly different between the three groups although there was a trend to increased values in CF patients. The ratio of AA to dihomo-gamma linoleic acid (DHGLA) (an indicator of Δ5-desaturase activity [1]) was decreased in CF patients, but not in the CF transplant group (results in Table 3.3).

Table 3.3: Ratios between selected fatty acids.

Median (IQR)	Control	TX	CF	Cntrolo versus TX (p)	CF versus TX (p)	Control versus CF (p)
AA/DHA	4.57 (0.98)	4.02 (1.81)	6 (2.53)	0.22	0.06	0.057
AA x DHA	73446 (73553)	109886 (104449)	45697 (52034)	0.49	**0.009**	**0.004**
MA/AA	0.02 (0.02)	0.04 (0.03)	0.06 (0.09)	**0.004**	**0.019**	**<0.001**
AA/DHGLA	4.81 (2.65)	4.52 (0.88)	3.73 (1.31)	0.38	0.12	**0.024**

Discussion

In our small cohort of CF patients we corroborate the frequently observed changes in serum fatty acid profile in CF patients [1;8-14]. There were decreased levels of LA, AA, DHA and increased levels of MA in our CF patients (all DF508). The exact etiology of these changes in plasma PUFA state in CF patients remains unknown. In this study, we compared a group of 11 lung transplanted CF patients with an age-matched, genotype matched group of 22 CF patients with comparable biometric data and comparable fish intake and a healthy control group. We demonstrated, for the first time, that after lung transplantation there is a more normal fatty acid profile (especially the poly-unsaturated fatty acids). Indeed, in our 11 CF patients (all DF 508) that underwent a lung transplantation (longer than one year ago), the plasma levels of LA, AA and DHA became comparable to healthy controls and MA levels decreased toward more normal values.

The mechanisms leading to the abnormal PUFA profiles are elusive. Numerous not mutually excluding hypotheses exist. In the setup we used, we can partially break the interdependency of pulmonary disease, pancreatic insufficiency and nutritional state with regard to genotype. All patients were pancreatic insufficient and remained so after lung transplant. So, decreased uptake seems unlikely to be an explanation, which is in accordance with previous findings in well-nourished CF patients [11]. In fact, the dietary intake was rather less in the transplanted group despite the better fatty acid profile.

Other proposed mechanisms are: decreased delta-5-desaturase activity in CF [16] (which fits with our finding of lower AA/DHGLA in CF patients (without transplant) compared to controls but could also be a sign of increased LA to AA conversion [17]); increased use of fatty acids as a source of energy [18] (which fits with the increased energy intake in CF patients compared to the transplanted patients although there was no difference in nutritional state expressed as BMI z-score); increased lipid turnover in membranes [19] and production of eicosanoids, possibly related to an exacerbated inflammatory state [20].

Lungs are in any case the most affected organs in CF and the number one cause of death in patients with CF. Therefore, we hypothesized that they could also play a central role in the development of abnormal fatty acid profiles. Moreover, there are some clues in this direction. Bronchial secretions from CF patients contain more AA as compared to three control groups (of healthy, chronic bronchitis patients and *P. aeruginosa* infected patients without CF) [21]. Furthermore, FEV1 has been related to CF-EFA abnormalities [14] and implicated in the

predisposition of CF lung disease [3]. Finally, in CF knockout mice lipid imbalances were detected in CF regulated organs as for instance in the lungs with decreased DHA levels and increased AA levels [5]. DHA supplementation could also block *Pseudomonas* endotoxin-enhanced lung inflammation in the same model [5].

By performing lung transplantation, and thereby normalizing PUFA state, we show that CF lungs are the major contributor to abnormal fatty acid state. However, in doing so there are three major changes: firstly the genotype changes partially, as 100% of the right cardiac output (in case of a double lung transplantation) is exposed to the non-CF tissue in the pulmonary circulation. Secondly, there is possibly a change in inflammatory profile from CF-related infection to low degree of infection or inflammation (rejection) under the use of immunosuppressive medication. The latter is reflected by the observed trend in decrease in AA/DHA ratio (Table 3.3). Finally, all the secondary effects of the diseased CF lung on various organs, such as for instance mediated by hypoxia, can become corrected by successful lung transplantation.

Several lines of evidence indicate a role for the CFTR itself, in the fatty acid metabolism. First, the degree of PUFA impairment is independently associated with the genotype group (mutation class) [12]. Second, heterozygotes with almost no inflammation have values intermediate to CF patients and healthy controls [7;13]. Third, other sources of inflammation as upper respiratory tract disease or asthma do not present with changes in the same magnitude as in CF [7]. This was also the case in bronchial secretions from non-CF *P. aeruginosa* infected patients [21]. Fourth, similar changes in CF affected organs (lung, pancreas, ileum) have been demonstrated in CFTR knock-out mice [5]. Finally, in cell culture models, it was shown that CFTR dysfunction itself leads to defective fatty acid composition (showing an increased conversion form LA tot AA) [17]. Nevertheless, inflammation is capable of changing PUFA composition [7], so it will probably also play a role in this complex metabolic alteration.

In this article, we chose to report our data in the form of µM rather than mol%. This is because we believe any biological effect modulated by the plasma is dependent on the concentration rather than the relation to the total fatty acid level. By calibrating the gas-chromatograph and mass spectrometer for each FA, we were able to determine the actual concentration (µM). Of course, ratios between selected fatty acids (Table 3.3) remain important and are unchanged by this. Moreover, since there was no statistical difference

between the total fatty acid level in the CF group and the control group, this makes no difference with regard to the published literature. It does become important when comparing the CF patients with the transplant patients as the levels in transplanted patients are significantly elevated compared to CF patients (Table 3.2). Nevertheless, when we would consider mole% we would also see an improvement in n-3 and n-9 series. However, the n-3 series do not significantly change in comparison to CF patients (See supplementary table).

A limitation to our study is that the CF lung transplant patients are not just CF patients with healthy lungs. They all take immunosuppressive medication which could have an effect on fatty acid state. Nevertheless, that this effect would lead to 'normalization' of EFA profile, apart from their effect on inflammation seems unlikely. Another limitation is that we choose to measure only total plasma fatty acid levels. However, these have shown to have a good relationship to lipid profiles in cell membranes of CF affected tissue and those obtained in red blood cells [1;5;11]. Finally, we did not measure EFA profile before and after transplantation, so we can not state that lung transplantation reverses abnormalities in EFA profile, only that a cohort of lung transplant CF patients has a more normal EFA profile than a non-transplanted cohort. However, the existence of a selection bias with patients with a better EFA profile undergoing lung transplantation seems counterintuitive.

To summarize, in this study we showed that the CF lung is an important player in the development of an abnormal fatty acid profile. Rather than studying the effect of fatty acids on lung function (as in EFA supplementation trials) we did the reverse by documenting fatty acid profiles in a subset of CF patients that underwent lung transplantation. We showed that an evolution towards normal fatty acid profiles is to be expected.

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Conflict of interest

PW is an aspirant researcher for the FWO Vlaanderen. DC is a fundamental-clinical researcher for the FWO Vlaanderen.

There is no conflict of interest.

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Supplementary table: FA profile in mol%

Mol%	CO	TX	CF	Control versus TX (p)	CF versus TX (p)	Control versus CF (p)
Lauric acid (c12:0)	0.067 (0.164)	0.145 (0.289)	0.056 (0.169)	0.46	0.46	0.46
Myristic acid (c14:0)	1.307 (0.708)	1.654 (1.642)	1.467 (1.2)	**0.024**	0.18	0.25
Hexadecanal dimethyl acetate (c16:0)	0.312 (0.142)	0.235 (0.098)	0.202 (0.12)	0.10	0.23	**0.001**
Palmitic acid (c16:0)	24.106 (3.037)	25.589 (3.519)	25.434 (2.745)	**0.036**	0.56	**0.023**
Stearic acid (c18:0)	7.267 (1.555)	6.972 (1.958)	7.262 (1.5)	0.10	0.13	0.85
Arachidic acid (c20:0)	0.205 (0.091)	0.152 (0.06)	0.153 (0.053)	**0.002**	0.99	**0.001**
Behenic acid (c22:0)	0.580 (0.173)	0.376 (0.271)	0.408 (0.179)	**<0.001**	0.61	**<0.001**
Lignoceric acid (c24:0)	0.449 (0.119)	0.307 (0.13)	0.286 (0.1)	**<0.001**	0.96	**<0.001**
Myristoleic acid (c14:1)	0.021 (0.438)	0.020 (0.211)	0.035 (0.164)	0.78	0.96	0.63
Palmitoleic acid (c16:1)	2.133 (0.853)	3.159 (3.108)	3.431 (3.402)	0.07	0.38	**<0.001**
Oleic acid (c18:1n-9)	20.293 (4.003)	21.281 (5.859)	22.799 (3.478)	**0.007**	0.61	**<0.001**
Vaccenic acid (c18:1n-7)	1.601 (0.526)	1.778 (1.077)	2.171 (0.818)	**0.017**	0.53	**<0.001**
Eicosenoic acid (c20:1n-9)	0.061 (0.072)	0.112 (0.148)	0.099 (0.076)	0.06	0.38	0.09
Erucic acid (c22:1n-9)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.59	0.53	0.95
Nervonic acid (c24:1n-9)	0.964 (0.314)	0.770 (0.306)	1.087 (0.41)	0.08	**0.032**	0.40
Alpha-Linolenic acid (c18:3n-3)	0.593 (0.288)	0.613 (0.337)	0.422 (0.306)	0.93	0.36	0.13
Eicosatrienoic acid n-3 (c20:3n-3)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.69	0.84	0.32
Eicosapentaenoid acid (c20:5n-3)	0.585 (0.429)	0.470 (0.701)	0.757 (0.355)	0.40	0.10	0.30
Docosatrienoic acid (c22:3n-3)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.00	1.00	1.00
Docosapentaenoic acid n-3 (c22:5n-3)	0.376 (0.174)	0.420 (0.277)	0.399 (0.278)	0.75	0.53	0.21
Docosahexaenoic acid (c22:6n-3)	1.644 (0.77)	1.624 (0.43)	1.176 (0.862)	0.90	0.17	**0.044**
Linoleic acid (c18:2n-6)	28.735 (4.768)	22.762 (9.545)	20.307 (5.198)	**0.004**	0.20	**<0.001**
Gamma-Linolenic acid (c18:3n-6)	0.306 (0.206)	0.311 (0.25)	0.473 (0.228)	0.99	**0.036**	**0.011**
Dihomo-gamma-Linolenic acid (c20:3n-6)	1.500 (0.537)	1.391 (0.463)	1.708 (0.615)	0.40	0.05	0.13
Arachidonic acid (c20:4n-6)	7.142 (1.741)	6.517 (1.242)	6.490 (1.734)	**0.044**	0.49	0.08
Docosatetraenoic acid (c22:4n-6)	0.114 (0.052)	0.125 (0.068)	0.150 (0.077)	0.28	0.38	**0.02**
Docosapentaenoic acid n-6 (c22:5n-6)	0.129 (0.075)	0.176 (0.248)	0.208 (0.141)	0.59	0.64	**0.029**
Mead acid/Eicosatrienoic acid n-9 (c20:3n-9)	0.131 (0.157)	0.219 (0.193)	0.402 (0.581)	**0.026**	**0.019**	**<0.001**

CF: cystic fibrosis, TX: lung transplant

Chapter 4: Non-invasive liver elastography (Fibroscan) for detection of cystic fibrosis-associated liver disease

Adapted from:

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Abstract

Background

Cystic fibrosis-associated liver disease (CFLD) is the second cause of mortality in CF. The prevalence is estimated to be 26-45%, but sensitive diagnostic tools are lacking.

We investigated if non-invasive liver elastography (Fibroscan) could serve as a screening tool.

Methods

Fibroscan measurements were performed in 66 CF patients. Age-specific cutoff values were determined in a control population (n=59). The measurements were compared to clinical data, biyearly biochemistry and ultrasound.

Results

Fibroscan was easy to perform in this patient population. There were 14 patients (21%) with abnormal liver stiffness measurements.

Liver stiffness was significantly increased in patients with clinical CFLD (11.2 kPa versus 5.1 kPa), biochemical CFLD (7.4 kPa versus 5.4 kPa) or ultrasonographical CFLD (8.2 versus 4.3 kPa) ($p<0.02$ for all).

Conclusions

Fibroscan is an objective measure and is easy to perform in CF patients, even in children and could provide a valuable tool to detect, and quantify CFLD.

Introduction

Cystic fibrosis associated liver disease (CFLD) is an upcoming health issue. Emerging during the second decade of life, it leads to significant morbidity and decreased quality of life in a selected population of young CF patients [1;2]. Moreover, it is the second most common cause of CF-related mortality [3].

The true prevalence of CFLD is difficult to estimate because the lack of sensitive and specific diagnostic tools. Based on the currently available clinical, biochemical and radiological methods the prevalence of liver disease is estimated at 26 to 45% [1;2;4]. However, these prevalence rates are likely an underestimation since in older autopsy studies focal biliary cirrhosis is reported in 72% of the cases [5]. Risk factors for the development of CFLD are also a matter of debate and might include male sex [4] (although not found in [2;6;7]), CFTR genotype [4;6;8] (not found in [1;2;7;9]), age at diagnosis of CF [9] or age at evaluation [6;8], meconium ileus [1;4;9] (not found in [2;6-9]), severity of pulmonary disease [7;9] (not found in [1;6]), pancreatic insufficiency [1;6] and height or weight [7;9] (not found in [6]).

In modern hepatology, non invasive tools for the diagnosis of presymptomatic liver disease are needed in order to prevent, if possible, progression of fibrosis with specific medical therapies. Furthermore, there is also a continuous search for minimally invasive, sensitive and quick detection of clinically important liver disease in order to start screening for esophageal varices, hepatocellular carcinoma and to prevent other complications of chronic liver disease. Although liver biopsy remains the gold standard, limitations are considerable, including patient discomfort and rare but serious complications such as bleeding or pneumothorax and a mortality rate of 1/10,000 to 1/12,000 [10]. Moreover, only 1/50,000 of the liver volume is investigated resulting in sampling error in focally distributed liver disease as CFLD [10]. Alternative attempts to diagnose and follow-up the liver disease range from routine biochemistry and calculated scores to surrogate fibrosis markers in serum, hepatic clearance tests, various imaging techniques and more recently the use of non-invasive transient elastography (Fibroscan ®) [11].

Although more than 55 studies have validated elastography in patients with hepatitis B, C, primary biliary cirrhosis (PBC) or sclerosing cholangitis (PSC), non-alcoholic steatohepatitis (NASH), haemochromatosis and post-transplant (for a meta-analysis see [12]), only one study

included patients with cystic fibrosis (n=42) [13]. However, this study did not include a separate analysis of the results in CF patients and did not take into account the special characteristics of CFLD as the main objective was to validate the use of the Fibroscan device in children. Additionally, there are only 3 studies in the paediatric population evaluating and validating the Fibroscan in the detection of liver fibrosis [13-15]. As CFLD does develops early in life and new cases after 20 years of age are rare [1;2], this mainly paediatric age group is the most interesting for screening and diagnosing CFLD. Early diagnosis allows a timely start of ursodeoxycholic acid therapy [7] or could at least be used to monitor the evolution of CFLD.

In this study we evaluated the possible role of Fibroscan in the early detection and screening for CFLD. The aim was to evaluate the diagnostic accuracy compared to other diagnostic tools as well as the relation of the liver stiffness to risk factors for CFLD.

Patients and methods

Patients

Sixty-six CF patients followed at the CF clinic at the university hospital Gasthuisberg, (Leuven, Belgium) were scanned consecutively from September 2006 to October 2007. Because of the lack of published normal paediatric reference ranges, a control group consisted of fifty-nine children attending the pulmonology clinic and eighteen healthy hospital staff members. None had a medical history indicative of any kind of liver disease or CF according to the patients/parents and to their medical files.

The study was approved by the local ethics committee and (parental) informed consent was obtained for all patients.

Fibroscan measurements

Liver stiffness was assessed by transient elastography (Fibroscan, Echosens, Paris). At least 10 measurements per patient are obtained, using the standard probe. Median values and interquartile range (IQR, kPa) are reported (for additional technical details see [15]). A success-rate of at least 60% was considered necessary. In the paediatric population special care was taken in order to make sure there was no A-shaped wave on the elastogram which indicates an incorrectly accepted (non-automatically rejected) measurement leading to an overestimation of the stiffness produced by influence of the surrounding rib bone and soft tissue [13].

Study design

At the time of the Fibroscan measurements, age, gender and weight, length, BMI and their respective z-scores (using recent Flemish reference values) were collected. A clinical questionnaire was filled out by the attending physician recording the presence or absence of hepatomegaly (>2cm below costal margin), splenomegaly, jaundice, icteric sclerae, spider angiomata, liver palms, itching or scratch marks, ascites or encephalopathy. The physician was asked to indicate on a visual analogue scale what the probability was that the patient had liver cirrhosis, based on the clinical examination and on previous biochemistry results.

Clinical liver disease was defined as the presence of hepatomegaly or splenomegaly [3].

Following data were collected from the CF patient's files: CF mutations (classified in three groups: F508del homozygous, two class 1-3 mutations and at least one class 4-5 mutation), pancreatic state (sufficient: stool elastase above 200 µg/g stool or insufficient: overt steatorhea without pancreatic enzyme intake (fat absorption less than 90% on 3 day faecal fat balance) or stool elastase below 100 µg/g stool), history of meconium ileus, ursodeoxycholic acid intake (never, persistently or intermittently), chronic Pseudomonas aeruginosa colonization (present/absent: repeated isolation of Pseudomonas aeruginosa (at least 3 positive sputum samples, with at least 1 month interval over at least a 6 month period) [16]).

Liver tests (AST, ALT, alkaline phosphatase, bilirubin and gamma-GT) from all CF patients from January 1996 to July 2007 were studied and patients with persistently elevated liver tests were identified (3-6 months, 1.5 times age-dependent upper limit of normal). **Biochemical liver disease** was defined as the elevation of 2 of these tests [3].

The North-American cystic fibrosis foundation (**CFF**) consensus workgroup defines CFLD as the presence of either clinical or biochemical liver disease [3].

Fibroscan liver disease was defined as a result above the age-related upper limit of normal liver stiffness. Information was collected from the clinical files to assess the relation between the obtained stiffness measurement and:

- the six-monthly CF routine blood analysis closest to the Fibroscan was assessed (white cell count and differentiation, red cell variables, platelet count and serum values for electrolytes, albumin, C-reactive protein, AST, ALT, GGT, PT, vitamin A, vitamin E, 25-OH-vitamin D, haemoglobin A1C, total cholesterol, IgG and IgE). The AST/platelet ratio index (APRI score), a non-invasive marker of fibrosis or cirrhosis, was calculated according to Wai [17].

- the lung function tests (FVC, FEV1 expressed as % predicted for sex, height and age according to Knudson, within 3 months of the liver stiffness measurement).

- the ultrasound findings (the presence or absence of hepatomegaly or splenomegaly (compared to age-related reference variables) and the Williams score, routinely calculated at our institution [18], within 6 months-1year of the liver stiffness measurement). The Williams score ultrasonographically evaluates the hepatic parenchyma, the liver edge and the degree of periportal fibrosis. On each of these three items a score of 1-3 is assigned: the hepatic parenchyma (1: normal, 2: intermediate abnormal or 3:irregular), the liver edge (1: smooth or 3: nodular (2 is never assigned)), the periportal fibrosis (1: absent, 2: moderate, 3: severe).

The scores of each of these items are added into one number from 3 (perfectly normal) to 9 [18]. **Ultrasonographic liver disease** was defined as a Williams score of at least 4/9 (i.e. intermediate coarse to irregular liver parenchyma, liver edge nodularity and/or moderate to severe periportal fibrosis) [18].

- the liver biopsy findings if obtained

- the presence or absence of esophageal varices if a gastroscopy was performed within two years of the liver stiffness measurement.

Statistical analysis

SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All results are expressed as means ± standard deviation. For differences between two continuous variables in two (or more) groups the Mann-Whitney U (or Kruskal-Wallis) test was used. To compare categorical variables Fisher exact test (2-sided) was used. For correlations within a group the Spearman Rank Correlation coefficient was used. A p-value <0.05 was considered statistically significant and a p-value <0.10, was considered as a statistical trend. Shapiro-Wilk test was used to assess normality of the Fibroscan results in the control population (a non-significant p-value indicates normality) and age groups and their specific normal value cutoffs were defined post hoc using age-liver stiffness scatter plots and Mann-Whitney U tests.

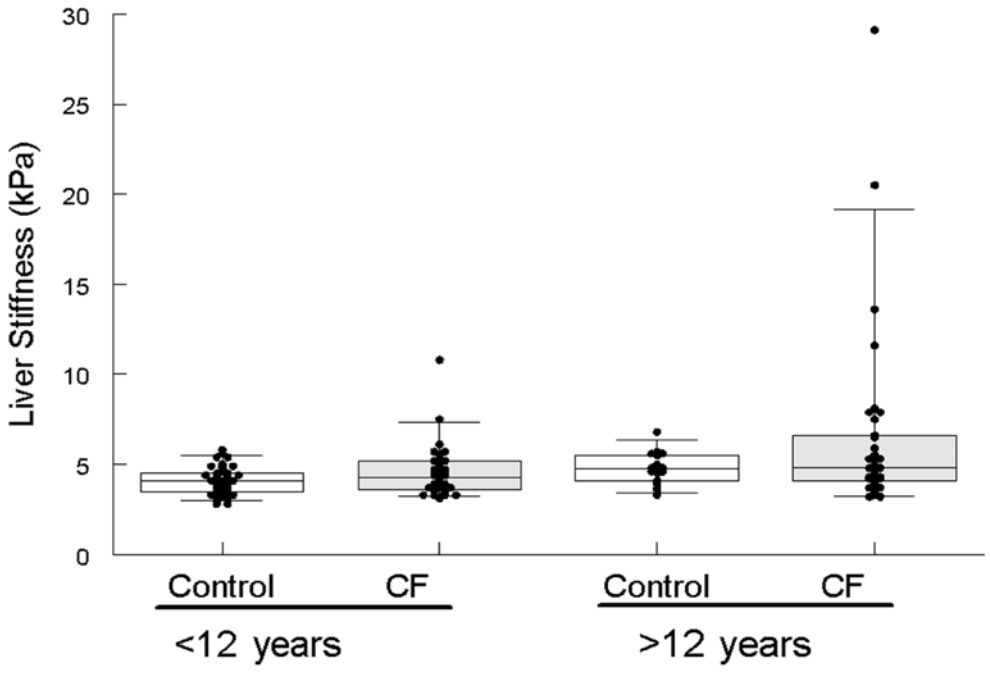
Sensitivity, specificity, positive predictive likelihood ratio and negative likelihood ratio were calculated. ROC curves with 95% confidence intervals (CI) were calculated to assess the diagnostic accuracy for the detection of CFLD.

Results

Control group

The control group (n=59) consisted of 26 male and 33 female subjects with a mean age of 10.2 ± 3.7 y (41 patients < 12y, 18 patients 12-18y). Fibroscan results did not differ between males and females or did not correlate with length, weight, BMI or their respective z-scores. However, there was an age-dependent increase in liver stiffness with higher values in the age group 12-18y (n=18) compared to the age group below 12 y (n=41) ($p<0.0001$, see Figure 4.1). As liver stiffness measurements were normally distributed within both groups (<12y $p=0.474$ and >12y $p=0.717$), the age-specific upper limit of normal value was determined as the average plus two standard deviations (i.e. the 98th percentile) and was 5.63 kPa for the <12y control group and 6.50 kPa for the >12y control group. As the liver stiffness measurements were not significantly different between the control groups 12-18y and >18y (n=18 in both groups, $p=0.815$), the same cut-off was used in the corresponding patient group (6.50 kPa).

Figure 4.1: Fibroscan results in controls and CF patients
Boxplots of liver stiffness (kPa) in the control groups (<12y (n=41) and >12y (n=18)) and in the CF groups (<12y (n=32) and >12y (n=34)).



Liver disease in cystic fibrosis

The CF group (n=66) consisted of 36 male and 30 female patients with a mean age of 13.6 ± 7.8y (32 patients < 12y, 24 patients between 12 and 18y and 10 patients >18y, for baseline characteristics see Table 4.1). Six patients (9%) had evidence of clinical CFLD (hepatomegaly or splenomegaly) and 7 (11%) had evidence of biochemical CFLD. Ultrasonography revealed hepatomegaly in 15 (23%) patients and splenomegaly in 16 patients (24%). 26 patients (39%) had clinical, biochemical or ultrasonographic CFLD.

Liver stiffness measurements in cystic fibrosis

The mean stiffnes in patients with CF was 5.63 ± 4.02 kPa with an IQR of 1.28 ± 1.01. There were 14 patients (21%) with abnormal liver stiffness measurements compared to the age-related normal values (for detailed characteristics of these patients see Table 4.2). Of these patients, 5 had clinical CFLD and 4 had evidence of biochemical CFLD. All but one had an abnormal Williams score (although she had clinical and biochemical CFLD, patient 1) and 12 had hepatomegaly or splenomegaly on ultrasound (compared to age-related reference values). The three patients (patient 6, 7 and 14) with the highest liver stiffness measurements (13.6, 20.5 and 29.1 kPa) had esophageal varices. One patient with a liver stiffness of 11.6 kPa (patient 8) underwent liver biopsy which showed periportal fibrosis and an occasional portoportal septum.

Liver stiffness measurements were significantly higher in the patient group with clinical liver disease (palpable hepatomegaly or splenomegaly) (11.07 ± 5.51 kPa (n=6) versus 5.08 ± 3.45 kPa (n=60) , $p<0.0001$), in the patient group with biochemical CFLD (7.40 ± 3.10 kPa (n=7) versus 5.42 ± 4.08 kPa (n=59), p=0.013), in the patient group with abnormal ultrasound (Williams score ≥4: 8.19 ± 5.96kPa (n=23) versus 4.27 ± 0.94kPa (n=41), $p<0.0001$) and also in the group with splenomegaly as detected on ultrasound (9.10 ± 6.78kPa (n=16) versus 4.54 ± 1.49kPa (n=48), $p<0.0001$). There was a significant correlation with the clinician's perceived probability of liver cirrhosis (R= 0.268, p=0.030).

Table 4.1: Baseline characteristics, suggested risk factors and relation to liver stiffness measurements

Liver stiffness values are reported (mean±standard deviation) or in the case of continuous variables the Spearman correlation coefficient.

		N (%)	Mean±Stdev	Liver stiffness measurement	
				(kPa)	*p*-value
CFLD definitions					
CFLD according to Consensus workgroup definition	Yes	11 (17)		8.83±4.79	**<0.0002**
	No	55 (83)		4.99±3.56	
US CFLD	Yes	23 (36)		8.19±5.96	**<0.0001**
	No	41 (64)		4.27±0.94	
Risk factors					
Gender	Male	36 (55)		5.44±3.00	0.221
	Female	30 (45)		5.84±5.02	
Genotype group	F508del/F508del	40 (60)		6.21±4.37	**0.009**
	2 class1-3 mutation	17 (26)		5.09±4.04	
	At least one class 4-5 mutation	9 (14)		4.02±0.58	
Age at diagnosis			1.6±3.1	−0.466	**<0.0002**
Age at hepatic work-up			13.6±7.7	0.263	**0.033**
Meconium ileus	Yes	13 (20)		8.15±6.92	**0.013**
	No	53 (80)		5.01±2.67	
Pulmonary function	FVC (%pred)		90.1±17.0	−0.059	0.649
	FEV1 (%pred)		81.9±23.9	−0.045	0.726
Chronic *Pseudomonas aeruginosa* colonization	Yes	15 (23)		7.81±6.61	0.062
	No	51 (77)		4.98±2.63	
Pancreatic insufficiency	Yes	58 (88)		5.86±4.23	**0.049**
	No	8 (12)		3.95±0.55	
Biometrics	Length (*z*-score)		−0.61±1.18	−0.055	0.659
	Weigth (*z*-score)		−0.59±1.14	−0.190	0.127
	BMI (*z*-score)		−0.27±1.00	−0.221	0.075

Table 4.2: Characteristics of patients with abnormal liver stiffness measurements

Abbreviations:

BMI: body mass index, Diagn: diagnosis, f: female, FEV1: one second forced expiratory volume, FVC: forced vital capacity, IQR: interquartile range, m: male, MI: meconium ileus, Pat Nr: patient number, PI: pancreatic insufficiency, %pred: percentage of predicted, *Ps. aer*: *Pseudomonas aeruginosa*, UDCA: ursodeoxycholic acid

Pat. Nr	age (yr)	Sex (m/f)	Stiffness (kPa)	IQR (kPa)	Clinical findings	Abnormal liver tests	Williams score, hepatomegaly or splenomegaly	Gastroscopy findings	FVC	FEV1	Weight	Length	BMI	UDCA	Genotype	Age at diagn	PI	MI	Chronic *Ps. aer.*
									% pred	% pred	*z*-score	*z*-score	*z*-score						
1	3.0	f	7.5	2.7	Hepatomegaly and splenomegaly	AST, ALT, Bilirubin, GGT	3, splenomegaly	–	–	–	−1.5	−0.6	−1.8	Sometimes	F508del/ F508del	0.3	Yes	No	Yes
2	6.9	m	5.7	1.2	Normal	None	5, hepatomegaly and splenomegaly	Normal	108.0	111.0	−0.8	0.0	−1.3	Never	F508del/ F508del	0.7	Yes	No	No
3	8.6	m	5.7	1.3	Normal	None	6, hepatomegaly and splenomegaly	–	106.0	103.0	0.9	0.7	0.8	Sometimes	F508del/ F508del	0.1	Yes	No	No
4	9.9	m	10.8	2.6	Normal	None	5, hepatomegaly and splenomegaly	–	90.0	93.0	−0.9	−0.5	−0.9	Sometimes	F508del/ F508del	0.0	Yes	Yes	No
5	11.5	m	6.1	1.7	Normal	None	4, normal liver and spleen size	–	85.0	88.0	0.6	−0.4	1.0	Never	F508del/ F508del	0.0	Yes	Yes	No
6	12.6	m	20.5	3.8	Hepatomegaly and splenomegaly	None	7–9, hepatomegaly and splenomegaly	Esophageal varices	76.0	48.0	0.1	0.0	0.2	Sometimes	F508del/ N1303K	4.3	Yes	No	No
7	15.5	f	29.1	6.5	Normal	None	9, liver cirrhosis, splenomegaly	Esophageal varices	102.0	102.0	−2.6	−1.7	−1.8	Sometimes	F508del/ F508del	0.0	Yes	Yes	Yes
8	15.7	f	11.6	3.4	hepatomegaly	None	5, hepatomegaly	Normal	64.0	51.0	−2.9	−2.6	−1.4	Sometimes	F508del/ F508del	0.2	Yes	No	Yes
9	16.1	m	6.6	1.9	Normal	None	4, splenomgaly	–	95.0	87.0	−3.7	−3.0	−2.3	Never	F508del/ F508del	4.8	Yes	No	No
10	16.6	f	7.5	1.3	Normal	AST, ALT, GGT	4, normal liver and spleen size	Normal	99.0	100.0	1.0	0.7	0.9	Never	F508del/ F508del	0.1	Yes	No	Yes
11	16.7	m	8.1	2.8	Normal	None	5, hepatomegaly	–	112.0	115.0	−1.0	−1.0	−0.5	Sometimes	F508del/ F508del	1.1	Yes	No	No
12	23.2	f	7.9	1.4	Splenomegaly	None	9, liver cirrhosis, splenomegaly	–	78.0	73.0	−0.7	−0.6	−0.5	Sometimes	F508del/ F508del	0.1	Yes	Yes	Yes
13	29.1	m	7.9	3.0	Normal	AST, ALT	4, steatosis, hepatomegaly and splenomegaly	–	73.0	39.0	0.9	0.5	0.7	Sometimes	F508del/ F508del	0.1	Yes	No	No
14	29.8	f	13.6	2.4	Splenomegaly	Alkaline phosphatase, ALT, GGT	9, liver cirrhosis, splenomegaly	Esophageal varices	64.0	33.0	−3.0	−2.8	−1.2	Sometimes	F508del/ F508del	0.0	Yes	Yes	Yes

Figure 4.2: ROC curve analysis for Fibroscan result, Williams score and APRI test against CFLD CFF definition

Area for Fibroscan 0.858 (95%CI: 0.738-0.977, p<0.001) versus area for Williams score 0.700 (95%CI: 0.512-0.888, p= 0.038) and APRI score 0.511 (95%CI: 0.299-0.724, p=NS)

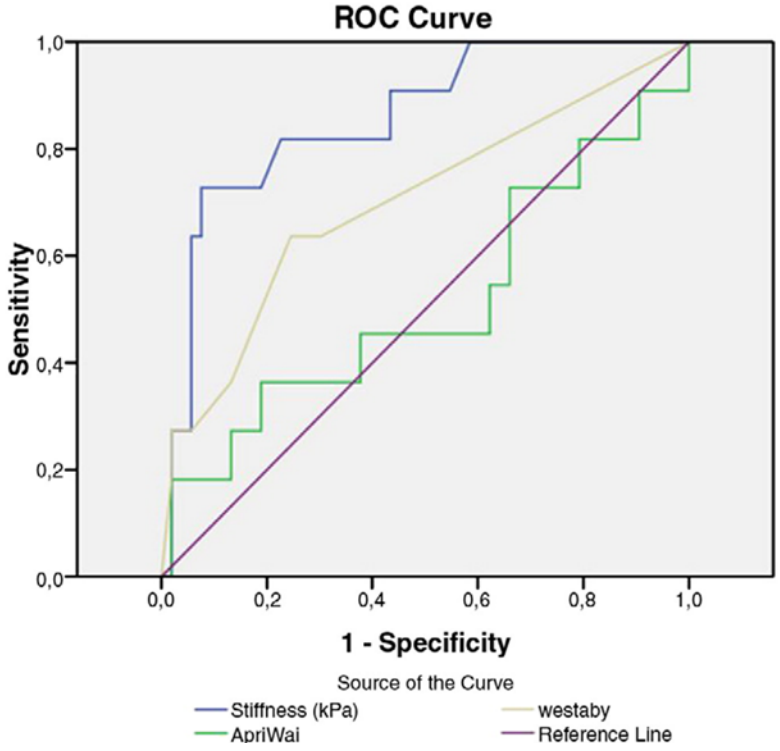


Table 4.3: Diagnostic accuracy of a) ultrasound and b) Fibroscan in the detection of clinical and/or biochemical liver disease

CFLD: Cystic fibrosis associated liver disease

CFLD definition used	Sensitivity (%)	Specificity (%)	ROC analysis		
			AUC	95% C.I.	*p*-value
A) Diagnostic accuracy of ultrasound					
Clinical CFLD	67	67	0.766	0.509–1.022	0.033
Biochemical CFLD	57	67	0.618	0.398–0.838	NS (0.312)
Clinical or Biochemical CFLD	64	70	0.700	0.512–0.888	0.038
Clinical and Biochemical CFLD	50	65	0.661	0.171–1.152	NS (0.44)
B) Diagnostic accuracy of Fibroscan					
Clinical CFLD	83	85	0.928	0.849–1.008	0.001
Biochemical CFLD	57	83	0.781	0.613–0.949	0.016
Clinical or Biochemical CFLD	63	87	0.858	0.738–0.977	<0.001
Clinical and Biochemical CFLD	100	81	0.923	0.837–1.009	0.043

Liver stiffness and the CFF consensus definition

Using the CFF consensus definition the Fibroscan has a sensitivity of 63%, a specificity of 87%, a positive likelihood ratio of 5.00 and a negative likelihood ratio of 0.42. ROC analysis revealed an AUC of 0.858 (see Figure 4.2 and Table 4.3). The Williams score for the detection of ultrasonographic CFLD has a lower AUC of 0.700. The ROC curve of the APRI score as non-invasive marker of liver disease was not significantly different from 0.500 (p=0.908). A more detailed comparison of the diagnostic accuracy of ultrasound and Fibroscan in the detection of CFLD in shown in Table 4.3. This shows that Fibroscan is not inferior or superior to ultrasound in the detection of CFLD in this patient population.

In the patient group with an abnormal Fibroscan result 7/14 patients do not have CFLD according to the CFF definition (patients 2-5, 7, 9, 11 see Table 4.2 and see Figure 4.3). However, all of these patients had abnormalities on hepatic ultrasound. Moreover, there was even one patient with suspected cirrhosis on ultrasound (patient 7).

On the other hand, there were 4 patients with CFLD according to the CFF definition (clinical or biochemical abnormalities) who had normal Fibroscan results (i.e. below the age-specific cut-off). One of these patients had clinical CFLD (clinical hepatomegaly, which could not be confirmed on ultrasound) and 3 had biochemical CFLD. All had normal hepatic ultrasound (Williams score < 4/9, i.e. normal liver edge, parenchyma and periportal findings).

Risk factors for CFLD

The traditionally recognized risk factors (genotype group, age, age at diagnosis, pancreatic insufficiency and history of meconium ileus) identified by ultrasound Williams score ($p<0.05$ for all), were also identified by the Fibroscan (See Table 4.1).

In addition, there was a significant negative correlation with the platelet count (R= -0.256, p= 0.038) and a positive correlation with the mean platelet volume (R= 0.329, p=0.007). There was no correlation with the APRI-score (p=0.169).

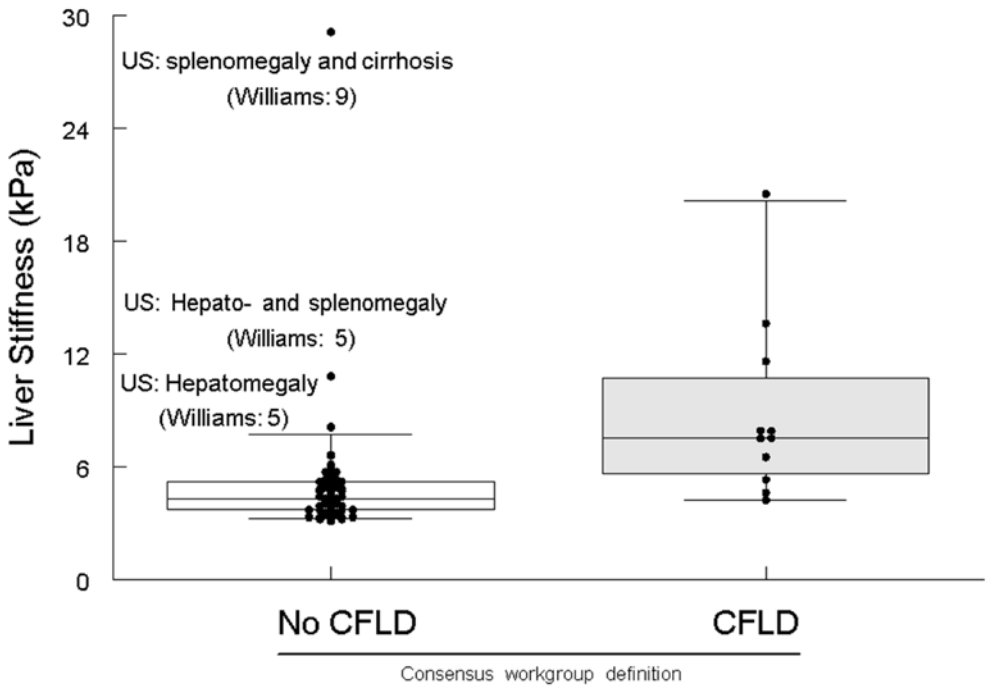
Discussion

Several studies have attempted to adequately screen for CFLD, but all have failed [19]. Developing new screening tools for the detection of CFLD is problematic, because there is no universally accepted definition of CFLD, classic non-invasive assessments of liver fibrosis are not validated for a paediatric population and several CF-associated processes can cloud the diagnosis (i.e. CF is a multisystemic disease whereby other processes e.g. infection and medication can modify classic non-invasive markers of liver disease (e.g. AST levels)) [3]. To our knowledge this is the first study to solely investigate the use of the Fibroscan in an unselected population of CF patients and relate the elasticity to the special characteristics of CFLD.

In this study we have shown that the use of the Fibroscan is feasible in CF patients. One examination (10 measurements) takes under five minutes and is feasible even in young patients. Moreover, in contrast to its use in paediatric NASH [15] the investigation is facilitated due to the pulmonary hyperinflation enlarging the intercostal space and the non-obesity of these patients.

Defining clinically significant liver disease is highly problematic [3]. It is defined according to the CFF definition on the basis of clinical findings or abnormal biochemistry [3]. It has been shown that diagnosing CFLD on the basis of clinical findings of hepatomegaly or splenomegaly has a sensitivity of only 42% and 57% and a specificity of 72% and 72% respectively compared to histology [20]. Likewise, an elevated ALT or GGT have a low sensitivity of 52 and 50% and a specificity of 77 and 74% respectively [20]. This makes it questionable to calculate the sensitivity or specificity compared to this definition (the gold standard) and could lead to an under- or overestimation of the diagnostic accuracy of the technique that is being evaluated. Nevertheless, the Fibroscan compares at least equal to ultrasound in a ROC curve analysis in reference to the CFF definition (see Figure 4.2). In the present series there is a sensitivity of 63% and a specificity of 87%. Six patients were detected that did not fulfill the definition of CFLD (CFF definition), while all of these patients did also have ultrasonographic evidence of CFLD and could have CFLD. One of these patients was categorized as probably having cirrhosis on ultrasound (patient 7, see Table 4.2).

Figure 4.3: CFLD CFF definition and liver stiffness measurements with selected ultrasound findings
US: Ultrasound



Although there is no clear difference in performance of the Fibroscan and of ultrasound in the detection of fibrosis in this particular study for this particular cohort of CF patients (see Table 4.3), Fibroscan has proven to have excellent inter- and intra-observer, intersite and interequipment agreements and overall superiority in the detection of fibrosis, when compared to ultrasound [21]. It is easy to learn, independent of professional training (i.e. a nurse could do it) [22]. The price of the Fibroscan apparatus is 70.000 euro, which is clearly more expensive than an ultrasound apparatus (15.000 euro for one probe, no doppler). Ultrasound has the advantage that it can detect more abdominal abnormalities than only liver fibrosis, although the signifance of these occasional findings is unclear [23].

No extensive anatomopathologic investigations were undertaken here. In CFLD, liver biopsy is known to be unreliable, due to the focal nature of CFLD [3]. So, biopsy should be guided by ultrasound in an attempt to obtain a representative sample, i.e. not in the most or least affected region [20]. Otherwise there would be an over- or underestimation of the total degree of liver disease. Moreover, since there are over 50 reports [12] confirming the close relation between the liver stiffness and degree of fibrosis (also in focal liver diseases characterized by biliary fibrosis as PBC and PSC) the question is raised whether it is ethical to perform a study

comparing Fibroscan to histology in a mainly paediatric CF population. A longitudinal follow-up of the here described cohort will clarify the evolution and the development of clinical CFLD in the patients with abnormal liver stiffness measurements.

It should be noted that all examined patients were presenting at the consultation in their usual state of health. This is important, as it is known that liver stiffness measurement in the setting of acute hepatitis (with a flare of transaminases), acute liver damage or extrahepatic cholestasis in jaundiced patients gives rise to an overestimation of the degree of liver fibrosis [24] .

Although not the main goal of this study, similar risk factors can be found using the Fibroscan compared to other modes of detection of CFLD (clinical, biochemical, ultrasonographical). We have found that genotype [4;6;8], age at diagnosis [9], age (at evaluation) [6;8], past medical history of meconium ileus [1;4;9] and pancreatic insufficiency [1;6] are risk factors in the development of CFLD in the studied population, which is in accordance with the current literature.

Currently, proof of effective therapies or preventive measures for CFLD is lacking. Partly this can be because of problems with the detection and definition of CFLD. Ursodeoxycholic acid is to date the only accepted treatment for CFLD [25], although improvement in clinically significant endpoints remains to be demonstrated [26]. In analogy to PBC, ursodeoxycholic acid could prove to be of benefit if given at sufficient dose and more importantly early enough. This requires early and reproducible diagnosis and non-invasive reliable follow-up endpoints. In the future, with the advent of antifibrotic therapies, fibroscan could also prove useful to screen for developing fibrosis.

In conclusion, CFLD is difficult to screen and diagnose with the current modes of detection. We have shown that Fibroscan is an easy, fast and non-invasive examination that could contribute to the early detection of CFLD. Further studies are needed to determine its role in the management of CFLD.

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Chapter 5: Noncirrhotic presinusoidal portal hypertension is common in cystic fibrosis-associated liver disease

Adapted from:

Witters P, Libbrecht L, Roskams T, De Boeck K, Dupont L, Proesmans M, Vermeulen F, Strandvik B, Lindblad A, Stéphenne X, Sokal E, Gosseye S, Heye S, Maleux G, Aerts R, Monbaliu D, Pirenne J, Hoffman I, Nevens F and Cassiman D; "Noncirrhotic presinusoidal portal hypertension is common in cystic fibrosis-associated liver disease." Manuscript submitted to Hepatology.

Abstract

Background and aims

Liver disease is the second cause of mortality in cystic fibrosis (CF). The current hypothesis gives a central role to bile inspissations and hence obstructed bile flow, but is unlikely to be the sole explanation. The aim of this study was to further elucidate the clinicopathological features of CF liver disease.

Methods:

39 liver specimens (30 needle biopsies, 9 explants), of 32 patients were analyzed and correlated with clinical characteristics. Fibrosis, biliary and vascular changes were evaluated according to prespecified criteria.

Results:

Cirrhosis was present in 9% (or 3/32 patients, only present in 3/9 explants). Classical biliary changes were present in 53% (17/32). The most striking histological abnormalities were portal vascular changes in 72% (23/32) and paraportal shunt vessels in 69% (22/32). The absence of cirrhosis in 5 patients with classical portal hypertension fits with noncirrhotic portal hypertension which was confirmed by hepatic venous pressure gradient measurements in 2 patients.

Conclusions:

Our results suggest that CF related liver disease predominantly presents as noncirrhotic, presinusoidal portal hypertension with portal venopathy. Shunting procedures to alleviate this portal hypertension should probably be considered before liver transplantation.

Introduction

Cystic fibrosis (CF) is the most frequent lethal genetic disorder within the Caucasian population, with an incidence of 1 to 2500-4000. Due to the current medical treatment, the median survival of CF patients has increased to more than 35 years [1]. Pulmonary problems are the main cause of death, but liver disease is also important and is the second cause of death [2] accounting for 3.5-7.8% [3]. Based on insensitive clinical, biochemical and radiological parameters, the prevalence of liver disease is estimated at 26 to 45% [4-6], but in older autopsy studies the characteristic lesion, focal biliary cirrhosis, is described to attain a prevalence of 72% [7].

The CFTR-gene (cystic fibrosis conductance regulator chloride channel) is expressed in the cholangiocytes that line the bile duct(ule)s and gall bladder, but not in the hepatocytes [8]. The current hypothesis on the etiology of CF related liver disease (CFLD) is that by decreased/absent CFTR function the water and sodium transport to the bile diminishes which leads to increased viscosity, diminished bile flow and increased concentrations of bile salts. This results in turn in inflammation and deposition of collagen around the bile ducts and portal tracts and to the 'characteristic' focal biliary and periportal cirrhosis which evolves to cirrhosis with portal hypertension [9;10].

However, there are some findings questioning this 'biliocentric' hypothesis. Histologically, bile inspissation is not common and does not correlate with the fibrosis noted. In fact, it is surprisingly infrequent (in less than 7% of patients with CFLD in one study [11]). Ultrastructural studies do not support the theory that biliary damage is the main pathogenetic factor in CFLD [9]. Clinically, the complications of portal hypertension (hepatosplenomegaly, variceal hemorrhage, ascites) are more frequent than with other types of biliary cirrhosis (up to 86% of patients with CFLD develop varices [3]). The classic stigmata of biliary cirrhosis are on the contrary less prevalent (itching, jaundice, erythema palmare, spider naevi). Biochemically, the cholestasis in CF is traditionally described as minimal to moderate [3], which contrasts with true biliary cirrhosis. The classic picture of cholestasis is even not present in CFLD except in neonatal cholestasis which is unrelated to the subsequent development of CFLD [3;12]. Biochemical tests in CF are poor predictors of the severity of liver disease [2;13]. Finally, treatment of patients with CFLD with ursodeoxycholic acid, has only shown to be useful in patients with abnormal biliary drainage on scintigraphy [14]. This suggests that mechanisms other than bile duct obstruction may play a role in the pathogenesis

of CFLD [15]. All these clinical, biochemical and therapeutic findings suggest that an additional explanation of CFLD is needed. Overall, portal hypertension is the main issue and cause of hepatic death while hepatic failure is a late event that is aggravated by bleeding episodes [3;13]. Even at the time of liver transplantation there is only a mild to moderate stage of cirrhosis [16].

Portal hypertension without cirrhosis is called noncirrhotic portal hypertension (NCPH). It presents with portal hypertension out of proportion to the histologic, clinical, biochemical liver disease [17]. The presence of portal hypertension in the absence of cirrhosis suggests a vascular component in the etiology of CFLD [18;19].

In this work we explored the presence of presinusoidal obstruction in portal venules in patients with CFLD.

Materials and methods

Patients

We studied 32 patients with CFLD of whom liver histology (39 specimens) was available. Fot this purpose, twenty-four biopsies in 18 patients from a previous study from the CF center from the Department of Pediatrics, Sahlgrenska University Hospital (Göteborg university, Göteborg, Sweden) were re-examined [4]. In addition, six biopsies in 6 patients obtained from the CF center, University Hospitals Leuven (Leuven, Belgium), were added. Finally, more advanced liver disease was studied on larger biopsy specimens obtained from 9 explant livers in 9 patients (one of whom already had a previous biopsy available), 4 from the CF center from the University Hospitals Leuven and 5 from the liver transplant center, Cliniques St Luc (Université Catholique de Louvain, Brussels, Belgium).

Written informed consent was obtained and current work was approved by the respective local ethics committees.

Following data were collected: genotype, sex, height, weight, body mass index, history of meconium ileus, age at CF diagnosis and liver biopsy, indication for biopsy, intake of ursodeoxycholic acid, presence/absence at time of biopsy of: pancreatic insufficiency, chronic *Pseudomonas aeruginosa*, *Burkholderia cepacia* or *Stenotrophomonas maltophila* infection, pulmonary function and biochemistry (levels of transaminases, gamma-GT, alkaline phosphatases, albumin, coagulation (prothrombin time or international normalized ratio), platelet counts and white cell counts) and presence or absence of splenomegaly on ultrasound. Where available, information of endoscopy (presence/absence of esophageal varices) and invasive (transjugular) portal pressure measurements was collected. All results are expressed as mean ± standard deviation.

Histological analysis

All liver tissue specimens had been prepared according to routine methods and were studied by two experienced liver pathologists (LL & TR) blinded to the patients' clinical data.

All biopsies had at least five complete portal tracts, which was considered a prerequisite for sufficient quality. A portal tract was defined as foci within the parenchyma containing connective tissue (by Sirius Red stain) and at least two luminal structures in the connective tissue mesenchyme, each with a continuous connective tissue circumference [20].

The degree of fibrosis was evaluated with the Metavir F score (F0: no fibrosis, F1: portal fibrosis with no septa, F2: few septa, F3: numerous septa without cirrhosis, F4: cirrhosis). The degree of steatosis was scored as absent, mild (<33 %), moderate (33-66%) or advanced (>66%) and the type as microvesicular, macrovesicular or mixed.

'Classical changes' of CFLD were defined as focal biliary fibrosis with a ductular reaction (i.e. more than 2 bile ductules in one portal tract) combined with an inflammatory infiltrate (predominantly neutrophilic). The typical bile inspissations (defined as periodic acid-Schiff positive inclusions in the bile ducts) were actively searched for.

The following definitions were used for the portal vasculature changes: he number of portal veins was considered too low if the portal vein was absent in 40% or more of the complete portal tracts [20]; a portal vein branch was considered too small when it was less than twice the size of the accompanying hepatic artery branch of bile duct [20]; a portal vein branch was considered too large if it was more than four times the size of the accompanying hepatic artery branch or bile duct [20]; endothelialitis was defined as the presence of an inflammatory infiltrate directly beneath the endothelium.

As features of preclinical portal hypertension, paraportal shunt vessels were documented as previously defined [21]) and centrolobular sinusoidal dilatation was defined as a dilatation of the sinusoids present in the draining areas of more than 50% of the central veins in the biopsy sample.

Results

Clinical characteristics of patients with esophageal varices

On analyzing the clinical characteristics of the 32 patients, there were 9 patients followed at a CF center (all from the University Hospitals Leuven) in whom complete information on the presence or absence of esophageal varices was available. There were 5 patients with clinically significant portal hypertension at the time of biopsy/transplantation. These patients had suffered from variceal esophageal bleeding (n=2), had undergone multiple sessions of variceal banding (n=1) or had a previous or concurrent gastroscopy demonstrating esophageal varices (n=2). In these patients, surprisingly, histological cirrhosis was not present. Actually, in these 5 patients (6 biopsies) there were only variable degrees of fibrosis (F1 in two, F2 in one and F3 in three biopsies). Ultrasound showed patent portal veins. Therefore, in all of these cases, there was evidence of NCPH. (See supplementary Table 5.1at the end of this chapter)

On a detailed histological study of these six biopsies, abnormalities in the portal vasculature (responsible for the portal hypertension) became evident. Portal veins were either considered too small or absent in more than 57% of portal tracts of all specimens. Incomplete septal cirrhosis (ISC) (blind ending septae extending from the portal tract into the hepatic parenchyma) was evident in two [22]. Paraportal shunt vessels as a pathological sign of portal hypertension were evident in all (See supplementary Table 5.1 and Figures).

Moreover, invasive transjugular portal pressure measurement, performed in two of these cases (1 and 2), showed that the hepatic venous pressure gradients (i.e. the difference between the wedged hepatic venous pressure (after balloon occlusion) and the free hepatic venous pressure) were below the cut-off of clinically significant portal hypertension, despite the presence of esophageal varices in both (i.e. 9 mmHg and 5 mmHg are below the 10 mmHg limit [23]). This is diagnostic for a presinusoidal component, which is consistent with the portal venopathy observed in all of the cases.

Histopathologic study

These findings of noncirrhotic portal hypertension in the patients mentioned above prompted us to further analyze all liver biopsies and explants specimens available, to see if vascular changes were also present. A summary of the clinical/biochemical characteristics at the time of biopsy or transplantation in these patients is shown in Table 5.1. All patients had pancreatic insufficiency. There were no patients chronically infected with *Burkholderia cepacia* or *Stenotrophomonas maltophila*.

Table 5.1: Baseline characteristics of the study population

		Needle biopsy (number or mean ± stdev)	**Explant liver (number or mean ± stdev)**
Gender	Female	13 / 30	4/9
	Male	17 / 30	5/9
Mutations	DF508/DF508	14	7
	DF508 heterozygotes	15	1
	1112delT/1112delT	1	0
	unknown	0	1
Diabetes mellitus		5/30	2/9
Meconium ileus		1/30	2/9
Chronic infection with	Pseudomonas aeruginosa	9/30	6/9
Age at diagnosis		1.39 ± 2.06 y	1.42 ± 2.20 y
Age at biopsy		14.52 ± 9.80 y	14.18 ± 7.17 y
Ursodeoxycholic acid intake		22 yes/8 no	8 yes /1 no
Anthropometry (z-score)	Height	-0.10 ± 0.98	-1.479 ± 1.85
	Weight	-0.28 ± 1.06	-1.733 ± 2.35
	BMI	-0.26 ± 0.96	-0.81 ± 1.39
Spleen size	enlarged	6/30	7/9
Lung function (% of predicted value)	FVC	91 ± 18	71± 23
	FEV1	84± 23	59 ± 28
AST	U/L (nl <38)	37.6 ± 23.6	40.9 ± 16.2
ALT	U/L (nl <41)	37.3 ± 25.0	60.2 ± 95.5
Gamma-GT	U/L (nl <53)	178 ± 707	44.8 ± 33.7
Alkaline phosphatases	U/L (nl <720)	519 ± 236	318.0 ± 295.4
Bilirubin	mg/dL (nl <1)	<1	2.8 ± 4.8
Albumin	g/L (nl 35-52)	41.5 ± 3.8	32.98 ± 4.9
Prothrombin time	% (nl 70-150)	103 ± 20	NA
INR	(nl 0.8-1.2)	NA	1.27 ± 0.18
Platelets	10*9 /L (nl 150-450)	292 ± 116	82.88 ± 35.74
WBC	10*9 /L (nl 4.5-13)	6.9 ± 2.0	2.65 ± 2.76

NA: not available, nl: normal

Needle biopsy findings in CF patients

We investigated 30 biopsies from 24 patients with CFLD in more detail. Indications for biopsies were biochemistry, i.e. AST, ALT or GGT > normal reference range (in 8 biopsies), abnormalities on ultrasound (4 biopsies), abnormal biochemistry and abnormal abdominal ultrasound (7 biopsies) or a follow-up biopsy thereof (2y: n= 5; 5y: n=5, 10y: n=1).

The mean number of complete portal tracts was 7.8±2.5 per biopsy and the total number of portal tracts (both complete and incomplete) was 9.2±3.0 portal tracts.

In 60% of the biopsies there was no fibrosis (F0). Cirrhosis (F4) was never present (see Table 5.2). Some degree of steatosis was present in one third of the biopsies and it was always form the mixed type (micro-macrovesicular).

Classical changes (ductular reaction, neutrophilic infiltrate) were present in at least one portal tract in nine biopsies (30% of biopsies), in 1/8, 1/7, 1/5, 4/16, 5/6, 7/7, 8/8, 11/11 and 11/11 portal tracts. Bile inspissations were only seen in two patients.

There were striking vascular changes in 14/30 biopsies (47%). Portal veins were absent in at least 40% of the complete portal tracts in nine biopsies in 4/10, 2/5, 4/8, 4/8, 3/5, 7/11, 4/6, 5/6 and 6/7 portal tracts (Figure 5.1). Additionally the portal vein was considered too small in four biopsies (1/8, 1/16, 4/7 and 11/11 portal tracts, combined with the absence of portal veins in the first biopsy). Finally, the portal vein was considered too large in two biopsies (in 4/8 and 1/11 portal tracts; the latter biopsy also showed one portal tract without portal vein). Clear thrombosis of portal vein branches was not seen. Of note, there were 5 biopsies with these clear vascular changes that had no evidence of the classical changes.

Nodular regenerative hyperplasia (NRH, atrophy of zone 3 hepatocytes and in zone 1 hyperplasia with double layers of hepatocytes, without significant fibrosis) and endothelialitis (consisting of a lymphocytic infiltrate directly beneath the endothelium of the portal venule) was evident in two biopsies each.

Table 5.2: Histology of liver biopsies

		Total number of needle biopsies n (%)	Number of needle biopsies with vascular changes n (%)
Total number of biopsies		30 (100)	14 (100)
Degree of fibrosis	F0	18 (60)	5 (36)
	F1	3 (10)	3 (21)
	F2	4 (13)	2 (14)
	F3	5 (17)	4 (29)
	F4	0 (0)	0 (0)
Steatosis	None	20 (67)	7 (50)
	Mild	5 (17)	3 (21)
	Moderate	4 (13)	4 (29)
	Severe	1 (3)	0 (0)
Classical changes	Present	9 (30)	9 (64)
	Absent	21 (70)	5 (36)
Inspissations	Present	2 (7)	2 (14)
	Absent	28 (93)	12 (86)
Nodular regenerative hyperpasia	Present	2 (7)	2 (14)
	Absent	28 (93)	12 (86)
Endotheliatitis	Present	2 (7)	2 (14)
	Absent	28 (93)	12 (86)
Paraportal shunt vessels	Present	17 (56)	9 (64)
	Absent	13 (44)	5 (36)
Centrolobular dilatation (>50% of draining veins)	Present	8 (27)	4 (29)
	Absent	22 (73)	10 (71)

Vascular changes are: Absence of portal veins (in 40% or more of the portal tracts) in 9 biopsies, too large portal veins in 2 biopsies, too small portal veins in 4 biopsies (combined absence of portal veins in one biopsy)

Figure 5.1 : Absence of portal veins

Hematoxylin-eosin stain of 4 portal tracts from different liver biopsies of CF patients (original magnification 200x). None of these portal tracts have demonstrable portal vein branches. Bile inspissations are present in 1A (asterix) and 1 D (brown pigment). Note that 1B has no inflammatory infiltrate.

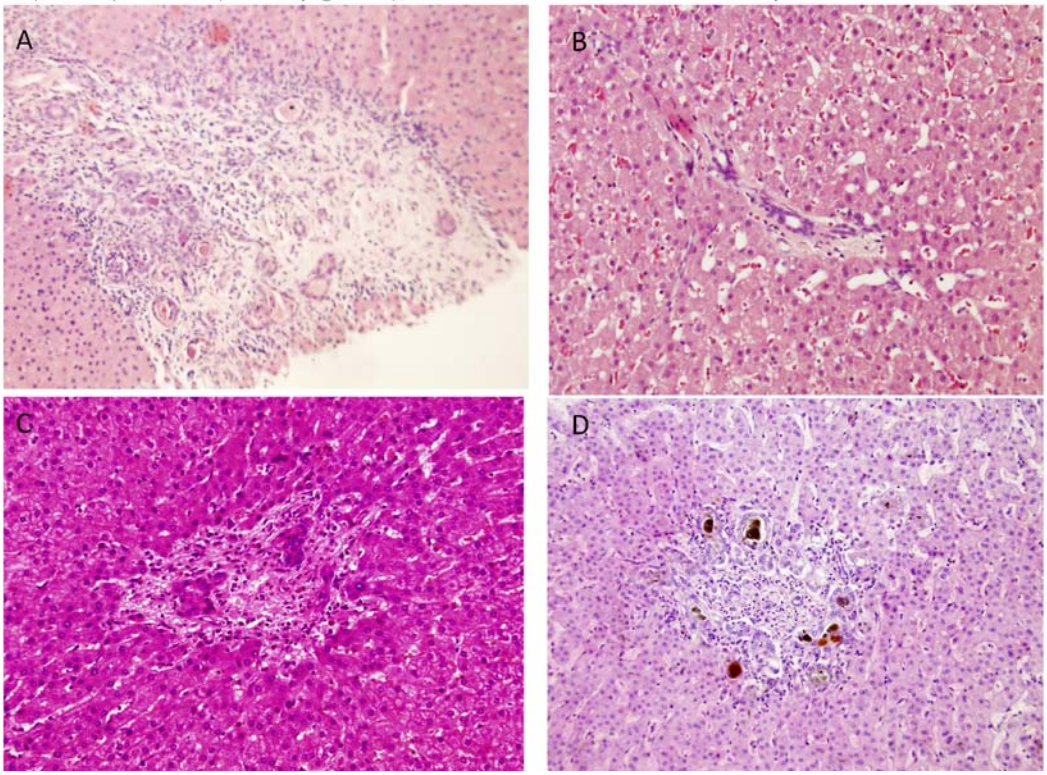


Figure 5.2 : Paraportal shunt vessels

Hematoxylin-eosin stain of 2 portal tracts from different liver biopsies of CF patients (original magnification 200x). Both have paraportal shunt vessels (asterixes) or paraportal thin-walled vascular channels that are a sign of portal hypertension.

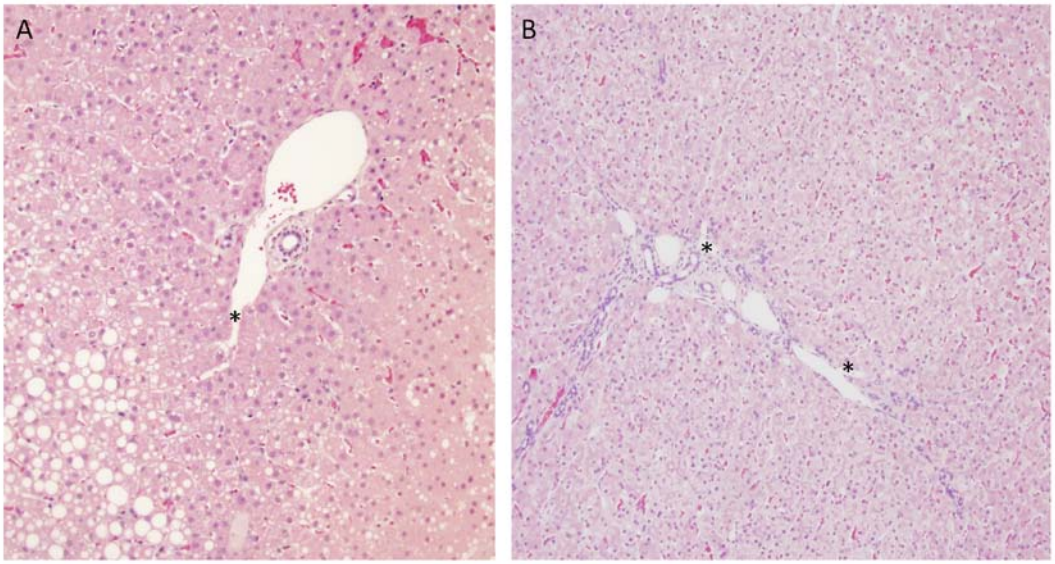
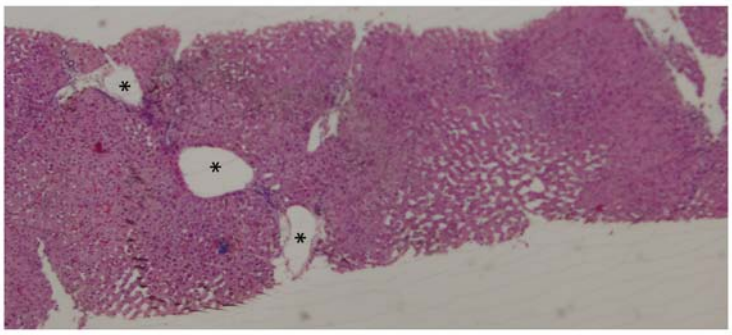


Figure 5.3 : Centrolobular dilatation
Hematoxylin-eosin (original magnification 40x) showing clear centrolobular dilatation and portal veins (*) that are widely dilated.



As histological (pre-clinical) signs of portal hypertension we found paraportal shunt vessels in 17 biopsies (57%): one paraportal shunt vessel in 9 biopsies, two shunt vessels in 1 biopsy, three in 2 biopsies, four in 1 biopsy, five in 2 biopsies and six in 2 biopsies (Figure 5.2). Of these, 9 had other portal vascular changes as described above. 8 had centrolobular vascular dilatation (present in the draining area of >50% the central veins) (Figure 5.3).

Liver explant specimens of CF patients

We investigated 9 liver explants specimens (Table 5.1, clinical characteristics). The indications for liver transplantation were mainly complications of portal hypertension (varices without bleeding were present in two, variceal bleeding in five, hepatic encephalopathy due to spontaneous portosystemic shunting in one, ascites in one and growth delay and delayed puberty in one). In two cases liver transplantation was combined with lung transplantation.

On histological examination only 3/9 livers had genuine cirrhosis (F4). In these biopsies disturbances in the microcirculation were to be expected (i.e. within the definition of cirrhosis [21]), thus portal phlebosclerosis or absence of portal venules was common. In none of these biopsies normal portal veins were recognizable (in two absence, in one too small calibers).

In two biopsies there was an alternation between cirrhotic areas and areas with less porto-portal septae (F3-4). In these biopsies classical changes were common (in 40% and 90% of the portal tracts respectively) but also absence of portal veins was common (in 60% of the portal tracts in each).

In three biopsies, staged as F3 (numerous septae without cirrhosis) the portal veins were absent in 70-80% of portal tracts. Two of these biopsies had clear paraportal shunt vessels as sign of portal hypertension. In one of these there was ISC. In this latter biopsy classical changes were not seen (only presence of ductular reaction but without neutrophilic infiltrate) (supplementary Table 5.1, case 3b and 4).

In only one explant liver with stage F2 fibrosis (few septa), frank thrombosis of the portal venules was observed. There were even calcifications within the portal veins and recanalisation of thrombi in others. NRH was evident (supplementary Table 5.1, case 5).

Discussion

In most CF literature and current clinical practice, portal hypertension (with splenomegaly, thrombocytopenia or esophageal varices) equals cirrhosis by definition. In our cases however, we saw that CFLD often presents as noncirrhotic portal hypertension (NCPH) with vascular lesions in the portal venules. For clinical and therapeutical purposes, we therefore propose to distinguish between CF related portal hypertension (CFPH) and CFLD with cirrhosis.

We described in detail five cases with CFPH, demonstrated by clear features of portal hypertension (esophageal varices) in the absence of a portal vein thrombosis and not associated with cirrhosis. In two cases transjugular invasive pressure measurements demonstrated a presinusoidal portal hypertension [23]. To our knowledge, this is the first study reporting on transjugularly measured (wedged) hepatic venous pressure gradient in CF patients. This finding was consistent with the histological changes in portal vein vasculature. Moreover, in this detailed retrospective study of 39 liver specimens (30 needle biopsies of patients with early CFLD and 9 explant specimens with advanced disease), these vascular changes were more prevalent (in more biopsies and in more portal tracts) than the classical changes described in CFLD. In other biliary diseases, as in primary biliary cirrhosis (PBC), the presence of this portal venopathy is also positively correlated with the presence of esophageal varices [24]. Finally, examination of the liver explants showed cirrhosis in only 3/9 patients (CFLD with cirrhosis) and variable degrees of fibrosis (CFPH) in the other patients. Other features as NRH and ISC (both characteristic of microvascular abnormalities [25]) could also be recognized.

NCPH is characterized by on one hand near normal liver function tests, hepatic venous pressure gradient not as high as in cirrhosis (namely <10mmHg), but on the other hand demonstrable varices, splenomegaly, decrease of one or more of the formed blood elements (e.g. thrombocytopenia), but absence of cirrhosis on histology. The lesion in NCPH is generally vascular, present in the portal vein, its branches or in the perisinusoidal areas of the liver [18]. These findings are similar to those we showed in the branches of the portal veins, indicating CFPH.

Other diseases commonly associated with NCPH are extrahepatic portal venous obstruction, schistosomiasis, sarcoidosis, congenital hepatic fibrosis, NRH and idiopathic portal hypertension or noncirrhotic portal fibrosis [18;25]. Interestingly, primary or secondary biliary cirrhosis in an early stage also present as NCPH [18;25]. Abraham et al. previously

showed, that actually all pre-cirrhotic chronic biliary diseases can be characterized by portal hypertension, leading to liver transplantation, while still in the noncirrhotic stage [26]. This is associated with the same features of portal venopathy and NRH. We have shown, for the first time, that CF can be added to this list of chronic (pre-cirrhotic) 'biliary' diseases (as previously demonstrated in primary sclerosing cholangitis (PSC), PBC, auto-immune cholangitis and secondary sclerosing cholangitis). Of interest, in CF patients (even without liver disease) PSC like lesions can often be revealed by magnetic resonance imaging [27].

As postulated in (early) PBC, it could be possible that the close proximity of the bile duct and the portal vein branch leads to a continuity of inflammation. As Wanless et al. stated, the members of the portal tract normally live in close proximity and when one member is affected by inflammation, the other members may become diseased as innocent bystanders (with spill-over of the inflammatory infiltrate from the bile duct to the portal vein) [28]. And indeed, we noticed that these lesions often (but not always) colocalize the classical changes of ductular reaction with a mainly neutrophilic infiltrate. This tendency of spill-over inflammation could represent the second hit leading to liver disease in a subset of patients with CF. For instance, increased inflammation in CF patients, possibly related to disturbances in fatty acid metabolism, might contribute to this [29].

It remains unclear if this is the sole mechanism leading to portal phlebosclerosis and secondary development of NRH or ISC. Thrombosis of a portal vein branch was seen in one case, where bile duct lesions were less prominent. It is not clear whether such thrombosis was primary or secondary to the portal hypertension. Blood platelets, the main actors in primary haemostasis, have been shown to be hyperactive in CF patients [30;31]. Furthermore, this hyperactivity has been implicated in the development of CF lung disease [30]. It remains unclear whether a similar role for platelets in CFLD can be postulated.

Finally, endothelialitis was seen in two biopsies. In CF, markers of vasculitis are often elevated [32]. This raises the question whether the inflammation of the endothelium could not be a primary event rather than a secondary, although clinically significant vasculitis is seldom seen in CF patients.

One strength of our study is clear definition of vascular abnormalities and detailed portal tract scoring. One limitation of our study however, is that we cannot determine which percentage of patients develop CFPH and how many may progress to CFLD with cirrhosis. It remains possible that CFPH is only a transitory stage (like in PBC/PSC). Additional hepatic venous

pressure gradient measurements in early and end-stage CFLD would be very informative. In any case, in our transplanted patients, portal hypertension was present in the same proportion as in the largest published European cohort (varices in 89% and bleeding in 42% [16]), suggesting liver transplantation was performed in a similar stage. It should likewise be noted that even in transplanted CF patients end-stage liver disease (and in our series cirrhosis) is rather seldomly seen, contrary to the portal hypertensive complications [16]. Furthermore, Lindblad et al. showed that histological changes in the liver biopsies are usually not progressive [4]. We showed that there is NCPH (CFPH) which is vascular and presinusoidal in nature in noncirrhotic CFLD.

Our findings could have important consequences for the management of at least a subset of patients with CFLD. Treatment of CFPH rather than treatment of CFLD with cirrhosis (by liver transplantation) is warranted in the patients without problems of liver synthetic function or detoxification. Therapy should be directed at the treatment of esophageal varices (prevention of bleeding and rebleeding). Contrary to the situation in cirrhosis, where the development of varices parallels the liver dysfunction, we have shown that varices precede liver dysfunction in CFPH.

There are no data available on the natural history of esophageal varices in CF. Currently, screening for the development of esophageal varices has not been advocated in children with CFLD [2], although bleeding is the main early complication [3]. Given the good liver function, mortality from rupture of esophageal varices is suspected to be lower compared to the mortality in patients with liver cirrhosis. This is similar as for other causes of NCPH.

There is no consensus for the use of beta-blockers for primary prophylaxis in patients with CFPH, moreover, they should be used carefully given the risk of reactive airway disease [2]. However, repeated variceal ligation under general anesthaesia could also have a deleterious effect on pulmonary function. So, the placement of transjugular portosystemic shunts or surgical shunts could be more beneficial in these NCPH CFLD patients. In CF, transjugular portosystemic shunting has been performed and published as cases or small case series [33]. Unfortunately, in these cases the wedge pressures were not determined/reported to demonstrate the presinusoidal etiology. This topic definitely requires further study.

In conclusion, although theoretically CFLD is a classic biliary disease, clinically, biochemically and histologically a vascular component and portal hypertension seem more important. Early CFLD presents as noncirrhotic, presinusoidal portal hypertension (CFPH). A

subset of patients with CFLD ultimately is referred for transplantation, but even in this group end-stage biliary disease is often not found. In our opinion, the management of these patients should aim at alleviation of the portal hypertension by shunting procedures rather than liver transplantation, but this remains to be studied in a formal prospective trial or a larger patient group.

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Supplementary Tabe 5.1: Five case of CF-related portal hypertension

Case, age, gender	1, 15 y, male	2, 19 y, female	3a, 12y, male	3b, 14y, male	4, 15y, female	5, 20 y, male
Diagnosis	N1303K/DF508, diagnosis at 3.5y	DF508/DF508, neonatal diagnosis	DF508/DF508, diagnosis at 7y	-Idem	DF508/DF508, neonatal diagnosis	DF508/DF508, diagnosis at 2y
Medication intake	Ursodeoxycholic acid, fat-soluble vitamins, pancreatic enzyme supplements and antibiotics	Fat soluble vitamins, pancreatic enzyme supplements, insulin, antibiotics, antifungals, proton-pump-inhibitors, inhaled cortisone, tranexaminic acid and supplemental orogastric tube feeding	Fat soluble vitamins, pancreatic enzyme supplements, ursodeoxycholic acid, aerosolized antibiotics and bronchodilators	- Idem	Ursodeoxycholic acid, fat-soluble vitamins, pancreatic enzyme supplements, proton-pump inhibitors, antibiotics, aerosolized mucolytics	Ursodeoxycholic acid, fat-soluble vitamins, pancreatic enzyme supplements, insulin and (aerosolized) antibiotics
Pulmonary status FEV1 (% predicted)	62%	30%	103%	89%	40%	21%, debilitating respiratory condition
Chronic pulmonary colonization	*Staphylococcus aureus* and *Hemophilus influenza*	*Pseudomonas aeruginosa* and *Stenotrophomonas maltophila*	*Pseudomonas aeruginosa*	*-idem*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
Pancreatic state	Exocrine insufficiency	Exocrine and endocrine (insulin-dependence) insufficiency	Exocrine insufficiency	-idem	Exocrine insufficiency	Exocrine and endocrine (insulin dependence) insufficiency
Hepatology:						
Biochemistry	- AST, ALT, GammaGT , alkaline phosphates and bilirubin: always normal - PT and albumin: always normal -thrombocytopenia, progressively declining over the years to 51,000 platelets/µL and white cell count of 3500/µL.	-AST, ALT and alkaline phosphatases:3 times ULN, Gamma-GT: 6 times ULN, bilirubin: normal -Prothrombin time: normal. Albumin: decreased (30.9 g/L, nl 35-52) - Thrombocytopenia of 78,000/µL	-AST, ALT <2 x ULN - GammaGT, alkaline phosphatases and bilirubin: normal -Prothrombin time and albumin normal -Thrombocytopenia of 53,000/µL	- Idem	-AST, ALT, GammaGT less than 1.5 times ULN - Bilirubin, alkaline phosphatases normal - Prothrombin time and albumin normal -Thrombocytopenia of 100,000/µL	-persistently normal liver tests (AST, ALT, alkaline phosphatase, gammaGT and bilirubin), -normal coagulation and albumin - thrombocytopenia of 92,000/µL.
Ultrasound	- Hepatomegaly 16,5 cm (nl 10-14 cm) with increased echogenicity, irregular contours and periportal fibrosis suggestive of liver cirrhosis - Portal vein was patent on ultrasound with a hepatopetal	-On ultrasound and MRI there was a macronodular liver. -Patent portal vein (on doppler: hepatopetal flow 21 cm/s) -Splenomegaly of 21x10 cm	-On ultrasound and MRI: inhomogeneous liver parenchyma with lobulated contours. -Patent (enlarged) portal vein with normal hepatopetal flow. -Splenomegaly (18cm)	-inhomogeneous, partial nodular liver parenchyma - Patent (wide) portal vein, normal hepatopetal flow - Splenomegaly (>20cm) -Small amount of ascites	- inhomogeneous nodular liver - Patent portal vein, hepatopetal flow (22cm/s) -Splenomegaly (15X8 cm) -Small amount of ascites	- Normal liver size, with lobulated contours, hyperechogenicity and hypertrophy of the left lobe - Wide portal vein with normal hepatopetal flow. -Splenomegaly (18cm) - Recanalization of the umbilical

	flow of 33cm/s - Splenomegaly of 17.8 cm (nl 8.8-12) - Recanalization of the umbilical vein and extensive network of collaterals on the ventral abdominal wall - No ascites	with calcifications. -Extensive collateral circulation via para-umbilical veins - Small amount of ascites.	-Some collaterals in spleen hilum - No ascites			vein - Some perisplenic free fluid
Endoscopy	Esophageal varices grade II-III (2 years earlier grade I)	Multiple sessions of variceal banding given the presence of esophageal varices.	At the age of ten years variceal bleeding with grade II varices. Subsequent eradication by sclerotherapy and ligation. At the time of biopsy: small esophageal varices disappearing with insufflations (grade I)	Varices grade I (disappearing with insufflations)	At the age of 14 y variceal bleeding with grade III varices with red spots. Subsequent eradication by sclerotherapy and ligation.	Esophageal varices not disappearing with insufflations (grade II)
Invasive pressure measurement (HVPG)	**Hepatic portal-venous pressure gradient of 9 mmHg (ICV pressure 18 mmHg, Wedge-pressure: 18 mmHg, Free hepatic veins pressure 13 mmHg)**	**Hepatic portal-venous pressure gradient of 5mmHg (ICV pressure 0 mmHg, Wedge-pressure: 5 mmHg)**	Not performed	Not performed	Not performed	Not performed
Biopsy indication **APO**	Abnormal ultrasound **- Periportal fibrosis (F1).** **- There is little/no inflammation.** **- Little or no cholate stasis (cytokeratin 7 stain) or ductular reaction but some dilated bile ductules with PAS-positive bile plugs** **- Mild (10-30%) mixed micro-macrovesicular steatosis** **- Important vascular changes: such as sinusoidal dilatation with the formation of paraportal shunt vessels (4/7 portal tracts) and too small portal veins (6/7 portal tracts)**	Abnormal ultrasound and biochemistry **- Numerous septae, without cirrhosis and less affected regions (F3)** **- Important cholate stasis with ductular reaction and bile inspissations in some portal tracts.** **- Mild (10-30%) steatosis, mixed type.** **- All portal veins were considered too small (11/11 portal tracts). Paraportal shunt vessels in 5/11 (mainly smaller) portal tracts.**	Abnormal ultrasound **- F1 (periportal fibrosis)** **- Ductular reaction without inflammatory infiltrate. Bile inspissations in one ductulus.** **- Mild steatosis (10-30%), mixed type** **-Portal veins too small in 4/7 portal tracts. Paraportal shunt vessels in 5/7 portal tracts.**	Liver transplantion **-F3, nodularity (incomplete septal cirrhosis)** **- Mild ductular reaction but without inflammatory infiltrates.** **Bile inspissations rather infrequent.** **- No steatosis** **- portal veins connecting with sinusoids (paraportal shunt vessels).** **- Absence of portal veins in 70% of portal tracts. In 5% of portal tracts too large portal veins.** **See supplementary Figure 5.1**	Liver transplantation **-F3, many portoportal septae but without cirrhosis** **-Mild ductular reaction with cholangiolitis and neutrophilic infiltrate.** **- No steatosis** **- Portal veins absent in 70% of portal tracts. Prominent presence of paraportal shunt vessels and centrolobular sinusoidal dilatation.** **See supplementary Figure 5.2**	Combined Lung-liver transplantation **-F2, incomplete septal cirrhosis with in some zones no septa and in others porto-portal and porto-central septa.** **- In most portal tracts the bile ductules are present with minimal ductular reaction. (minimal bile duct damage, seldom bile duct plugging)** **- No steatosis** **- Most portal tracts have either no portal vein or a portal vein that is too small. In other portal tracts there is thrombosis in the portal veins, with or without calcifications, with or without revascularization** **See supplementary Figure 5.3**

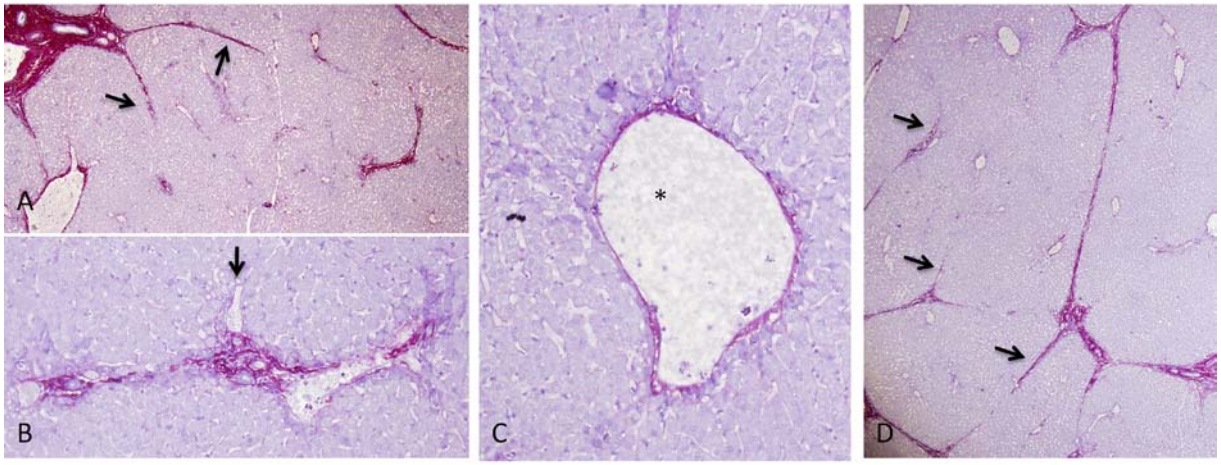
Supplementary Figure 5.1 : Case 3b

S5.1.A Original magnification 40X. Incomplete septal cirrhosis. Arrows: Thin incomplete septa radiating from fibrotic tracts.

S5.1.B Original magnification 200X. Arrow: paraportal shunt vessel (thin-walled vascular channels that are a sign of portal hypertension)

S5.1.C Original magnification 200X. Asterix: Dilated portal vein

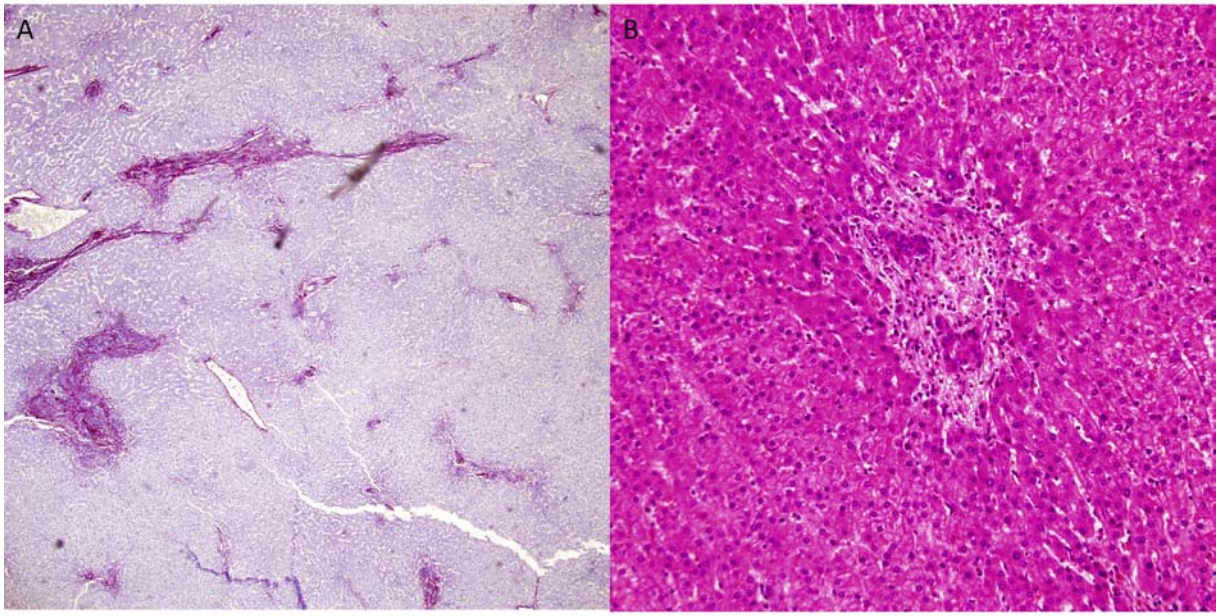
S5.1.D Original magnification 40X. Incomplete septal cirrhosis. Arrows: Thin incomplete septa radiating from fibrotic tracts.



Supplementary Figure 5.2 : Case 4

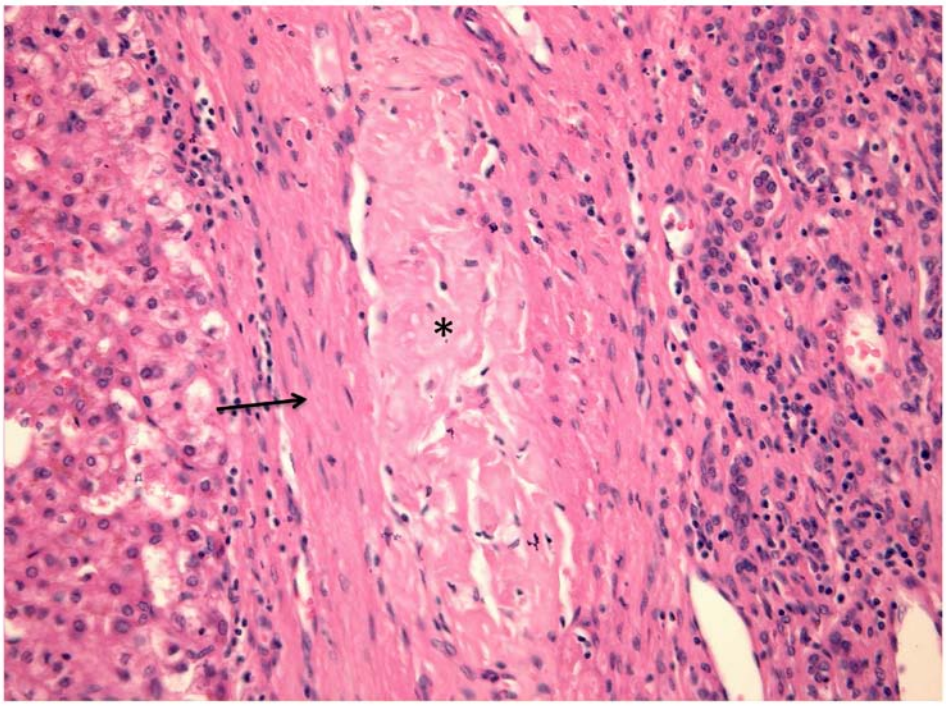
S5.2.A Original magnification 40x. Sirius red stain of case 4 showing an area with few portoportal septae. No evidence of cirrhosis

S5.2.B Hematoxylin and eosin stain of case 4. Original magnification 200x. Portal tract with absence of portal veins.



Supplementary Figure 5.3 : Case 5

Hematoxylin and Eosin stain. Original magnification 200X. Portal vein with phlebosclerosis. Fibrotic band is formed within the portal vein (asterix). The muscular layer of the vein is still recognizable.



General discussion: looking at the past with perspectives on the future

Present work

Platelets are important mediators in various diseases.

In this PhD thesis we have shown that cholestatic liver disease (in the rat model of common bile-duct ligation) inhibits platelets by a plasma-borne factor, which we further characterized as ADP-degrading enzymes. The platelet intrinsic functionality was found to be normal.

We further discovered that in CF, platelet aggregatory function is normal, but that inhibition thereof is decreased. Supplementation of DHA in vitro and in healthy volunteers, suggests that DHA deficiency could be the etiologic factor. In an additional project we have found that the diseased CF lung (and possibly all secondary changes associated with CF lung disease) can be held responsible for this deficiency.

In a last part we studied the pathogenesis of CF liver disease by case studies and histology. CF liver disease is difficult to diagnose. We have shown that the use of non-invasive transient liver elastography (Fibroscan) could constitute an objective assist in this area. Interestingly, we have discovered that CF related liver disease often presents as a non-cirrhotic presinusoidal portal hypertension rather than a cholestatic liver disease.

Cholestatic liver disease and platelets and, inversely, platelets and cholestatic liver disease

ADP-degrading enzymes inhibit platelet activation in a rat model of chronic cholestatic liver disease (common bile-duct ligation). Translation of these findings in similar human pathology (acute obstruction by choledocholithiasis or more chronic as for instance in tumors of the pancreatic head or more general in other cholestatic diseases as for instance PBC and PSC), awaits further research. However, as alkaline phosphatases and ectonucleotidases, both capable of degrading APD, are also greatly elevated in these diseases, the findings can be expected to be comparable.

Further characterization of the exact enzymes responsible for the degradation of ADP could lead to the development of inhibitors of these enzymes. Thereby, primary haemostasis could be improved or even normalized, an effect probably not attained by platelet transfusions as these new platelets would also be inhibited. This would be clinically useful as these patients often undergo surgery or invasive procedures.

Besides these findings of the effect of cholestatic liver disease on platelets, the reverse, the contribution of platelets to cholestatic liver disease is even a more intriguing field of research. Although platelet aggregation is inhibited, platelets are probably still capable of exerting their inflammatory and regenerative functions [126], as intrinsic platelet function is normal. Indeed, platelets have been shown to be important in liver inflammation and in liver regeneration [127]. Platelets could for instance contribute to the recruitment of leucocytes into the hepatic parenchyma, via interaction of their P-selectin receptors with leucocytes. Also, platelet-derived serotonin has shown to mediate liver regeneration [11].

Specific to cholestatic liver disease, various interesting observations in this field have been made. In a model of alpha-naphthylisothiocyanate-induced cholestasis in Sprague-Dawley rats, thrombocytopenia (induced by an antibody) led to decreased cholestatic liver injury. The elevations of ALT and bilirubin in the thrombocytopenic rats were less pronounced 24 hours after ANIT administration [128]. Also, in a model of 12 hour bile-duct ligation in mice, platelets were shown to contribute to liver injury by promoting leucocyte recruitment and deteriorating the microperfusion through the formation of aggregates and also by rolling and adhesion of platelets in sinusoids and postsinusoidal venules. Antibody-mediated platelet depletion led to a lesser increase in transaminases. Interestingly, blocking P-selectin interactions by another antibody had a similar effect without affecting platelet counts [10]. In a follow-up report from the same group, the use of a Rho-kinase inhibitor (inhibiting platelet functions) could similarly decrease this platelet-mediated deterioration of the microvascular perfusion of the liver [129].

One could believe platelets are detrimental to the cholestatic liver. However, in a very recent report, using a 10-day bile-duct ligation model in normal and thrombocytopenic mice (using thrombocyte-specific Bcl-xL knockout mice), platelets were shown to protect against liver fibrosis. Although thrombocytopenia led, also here, to lower ALT levels, there was more fibrosis. The presumed mechanism is that activated platelets secrete HGF which inhibits collagen production by activated stellate cells. And indeed, HGF administration alleviated

fibrosis in the thrombocytopenic mice [130]. Clearly this is an exciting field for additional research.

To close the circle and study the role of platelet function and platelet numbers on the development of liver disease, we attempted to influence platelet function or platelet counts in our cholestatic rats. We tried daily aspirin injection at normal and high doses, which did not provoke any differences in liver disease (as evaluated by biochemistry and degree of fibrosis on histology). Additional in vitro experiments with addition of aspirin to rat whole blood, however did not affect rat platelet aggregation, which is clearly different from what is expected based on human and mouse data and was hitherto unknown for rat. In a second batch of experiments, we injected a commercial polyclonal antibody raised against rat platelets, to obtain platelet depletion in our rat model, this to study the effect of platelet depletion on liver disease. We gave three injections, one every 48 hours, with increasing dose. More frequent injections were avoided, to prevent immune responses against the antibody that would confound the data. The antibody was indeed capable of provoking temporary platelet depletion, but also provoked reactive thrombocytosis with platelet counts rising to 2x normal counts afterwards. Since 14 days are considered necessary to obtain biliary cirrhosis in BDL rats [131], platelet depletion by means of antibodies was not feasible for this whole period. At the time of sacrifice (day 14) platelet counts in the BDL/platelet-depleted group (n=6) were still higher than in the BDL group (n=12). Moreover, biochemistry did not reveal any trend towards improvement or deterioration in liver tests. In a third batch of experiments, total splenectomy was performed to provoke thrombocytosis. To avoid the splenectomy in itself would be a confounder, we compared the effect of bile duct ligation in a group of rats, splenectomized 4d before BDL (thrombocytosis group, n=8), with a group of rats that had undergone splenectomy 1 month before BDL (normal thrombocytes group, n=8). However, both groups (4 days and 30 days splenectomy) developed equal and long-lasting thrombocytosis, contrary to what is reported in the literature [132]. The model therefore did not allow to study the effect of differences in platelet counts on liver disease, within a reasonable time frame. In a fourth batch of experiments, we used rat models with known platelet deficiencies. Although not emphasized in the first chapter, we also studied the liver of the fawn-hooded rats. Fawn-hooded rats have a storage pool deficiency, therefore providing us with a good model to study bile duct ligation in a model with defective platelets (no secretion of delta-granules). As presented in chapter one, we saw no differences in liver function tests. This does not necessarily mean platelets are not at all implicated in the disease

process, as it could still be that bile-duct ligation was a too drastic model, leaving no room to pick up subtle differences between the two experimental groups. We also studied bile duct ligation in Wistar Furth rats, a rat model with a known deficiency in platelet alpha granules (and hence an animal model for grey platelet disease [133]). Interestingly, after 14 days of bile duct ligation, we saw increased levels of AST and ALT in the Wistar-Furth rats compared to control BDL rats. Moreover, on histology there was an increased staining with Sirius red (a marker for the degree of fibrosis). However, a detailed literature search then revealed that under stress conditions in vitro, talin proteins in hepatocytes are involved in hepatocyte blebbing and subsequent cell death [134]. This talin (a cytosolic protein capable of linking integrins to the actin cytoskeleton) is however impaired in these Wistar Furth rats and therefore it was not excluded that the effect on hepatocyte necrosis we measured in vivo, was in fact caused by the underlying genetic defect in Wistar-Furth rats [135], rather than being secondary to the platelet defect they demonstrate. We finally concluded that studying the contribution of platelets to the development of biliary cirrhosis in the BDL rat model we used, had proven to be an impossible challenge. There probably are solutions to the problems we encountered, but time constraints did not allow us to pursue them any further. For instance, we would suggest using a mouse model, which is technically more difficult (need for pooling of blood from several mice to allow platelet function testing, need for microsurgery to perform BDL), yet not impossible. The literature reports on feasible mouse models for platelet depletion and thrombocytosis and there are interesting knock-out mouse models with dysfunctional platelets or abnormal platelet counts available. Additionally, using another model for cholestatic liver disease (less drastic than bile duct ligation, for instance alpha-naphthylisothiocyanate-induced cholestasis) might be more rewarding in the search for discrete changes in the evolution of liver disease, provoked by platelet changes.

CF, EFA and platelets and, inversely, platelets, EFA and CF

We have studied the influence of CF on platelet function and on EFA profile. Platelets are more sensitive to inhibition and we have documented a putative role for the EFA profile. Although supplementing the deficient DHA in healthy controls leads to increased inhibition, this remains to be studied in a DHA supplementation trial in CF.

Platelets are implicated in the inflammation of CF lung disease [92;94]. Increased platelet numbers correlate inversely with the decreased arterial oxygen pressure [136]. Furthermore,

increased thromboxane metabolites and soluble CD40 ligand, both mainly produced by platelets, correlate with the decreased pulmonary function [137-139].

Similarly, lung function is correlated with fatty acid abnormalities [140] and EFA deficiency is implicated in the predisposition of CF lung disease [82]. Additionally, we have shown that the diseased CF lung influences EFA profile and that certain EFA, such as DHA, can influence platelet function.

Whether platelet function, after lung transplantation in CF, normalizes due to normalization of EFA profile, remains unstudied and is an interesting field for further research. However, a carefully chosen control group, receiving the same immunosuppressants, seems necessary. The effect of immunosuppression on the inhibition of aggregation is hitherto unstudied.

DHA levels in the platelets themselves, were not different between CF patients and healthy controls in one study [141]. However, platelets are capable of incorporating DHA in CF patients upon supplementation [142]. Additionally, DHA has shown to influence platelets without incorporation in the platelet membrane, as normal platelet functionality returned upon removal of exogenously added DHA by washing the platelet suspension [143].

Abnormal PUFA profile comprises more than decreased DHA levels. Although the two most consistent features of the disturbed PUFA profile in CF patients appeared to be reduced circulating levels of LA (linoleic acid) and DHA. Supplementation of DHA can correct DHA deficiency but also LA deficiency through decreased formation of arachidonic acid in vitro (CF cell lines) [144] and improves the increased AA/DHA ratio (or the n-6/n-3 balance, determinant for the inflammatory profile) in vivo (CF patients) [145].

Whether these alterations are primary or secondary to the generalized inflammation remains to be shown [92]. Final proof of their importance in the CF physiopathology can only come from interventional trials or animal experiments.

Interestingly, DHA supplementation in CF knockout mouse models have shown reversal of pancreatic and ileal pathology [146]. Additionally DHA blocked *Pseudomonas* endotoxin-enhanced lung inflammation [146]. Evaluating more longterm DHA supplementation in a CF mouse model with a different genetic background demonstrated a significant amelioration of CFLD [83]. In CF patients the effect of supplementation is less clear. Although supplementation leads to improvement of EFA profiles and decreased inflammatory markers, therapeutic benefit is less convincing [78]. A Cochrane review does not recommend routine

use of n-3 fatty acid supplements [77]. Although the beneficial effect of DHA supplementation is attributed to an anti-inflammatory effect [83], we have shown, in healthy volunteers, it likewise affects platelet function. This could constitute another therapeutic mechanism.

Inversely, interfering with platelet function could represent a novel therapeutic approach. Trials with ibuprofen, a weak platelet antagonist, have shown beneficial effects on lung function. A Cochrane review states that high-dose ibuprofen can slow the progression of lung disease in CF [147]. The exact mechanism remains unclear, but decreased neutrophil delivery to the lung seems important [147]. It is not clear whether platelets play a role here. There are also some reports on low-dose aspirin in CF patients. They can decrease the production of inflammatory mediators (like TXA2) however, their therapeutic benefit was not evaluated [92]. The therapeutic potential of more selective platelet inhibitors (for instance clopidogrel, ticlopidine, tirofiban or abciximab) remains an interesting area fur further research.

CF and liver disease

Looking back on our work of the fibroscan in the detection of CFLD associated fibrosis, we could correlate the measured liver stiffness with ultrasound, clinical and biochemical characteristics in CFLD. These findings were later confirmed in a subsequent study on Fibroscan and CFLD by another group [148].

It was not considered ethical to perform liver biopsies in all the CF patients participating in this study. Moreover, due to the presumed focal nature of the disease, there is a high risk this would be non-contributive [106]. It is recommended that biopsy should be guided by ultrasound in an attempt to obtain a representative sample, i.e. not in the most or least affected region [120]. Therefore, we did not pursue a correlation between the liver stiffness and the anatomopathological degree of fibrosis.

In the second part of our research on CFLD, we have however shown that in CF, CFLD can present with portal hypertension to a greater degree than would be expected on the basis of the degree of fibrosis (NCPH). This is also the case in patients with NRH (another form of NCPH, with histology comparable to some of our cases of CFLD). In this disease, liver stiffness does not correlate with degree of fibrosis [149]. Its relation to portal hypertension remains to be studied [149].

In our study on CFLD we have suggested early disease is a form of NCPH (CFLD with portal hypertension), with important vascular changes. It remains to be determined which proportion of CF patients further develops CFLD with cirrhosis. As stated in chapter 5, it could still represent a transitory stage (like in PBC/PSC). However, documenting the nature of the CFLD in a given patient (only portal hypertensive versus cirrhotic) does have implications in the treatment (treatment of portal hypertension with beta-blockers, variceal ligation or shunts versus liver transplantation).

With regard to the rest of our work the logical question would be: 'Are platelets implicated in the pathogenesis of CF liver disease?'.

It is very interesting that supplementing DHA (which would increase platelet inhibition) in a mouse model of CF led to a decrease in liver disease [83]. Of course, other mechanisms (modulating eicosanoid-induced inflammation) could also play a role. The treatment of these CF knock-out mice with aspirin, or a more selective platelet antagonist could answer this question.

Four years ago, we envisioned a similar experiment. To this pursuit this, we worked together with prof H. de Jonge, Rotterdam, the Netherlands, to obtain a CF knock-out mouse model. However, histological analysis of the livers of these mice did not show any liver disease in our hands. Possible explanations were that the mice were considerably younger than their published counterparts that do demonstrate liver disease [150]. Other explanations were that these mice were bred in specific pathogen free circumstances and had a different genetic background.

As CFLD bears great similarities to NRH, it would seem interesting to study platelet function in NRH (and other forms of NCPH). As these diseases have vascular (sometimes thrombotic) changes in the liver, platelets could be implicated in the pathophysiology.

Studying platelet function in cholestatic (common-bile duct ligated) rats, although bearing resemblance to the 'biliocentric'hypothesis of CFLD seems not contributive to answer this. Animal models of nodular regenerative hyperplasia for example selenium-enriched diet in rats [151] or in a canine model of mucopolysaccharidosis type I [152] would be interesting. Currently, a study of platelet function in patients with histologically and endoscopically proven NCPH (mainly early PBC/PSC) has already been initiated.

Finally, additional suggestions for further research are the effect of correcting the EFA profile on CFLD (possibly mediated by platelets) and the influence of lung transplantantation (and thereby improvement of EFA profile).

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Summary

In patients with liver disease disturbances in platelet function may occur. Therefore, we studied platelet function in a rat model of liver diseases with diminished bile flow (bile-duct ligated rats). We found that platelets actually function normally, but they are inhibited by a plasma-borne factor. We showed that this factor is the presence of ADP-degrading enzymes (ADP normally stimulates platelet function).

Cystic Fibrosis is a hereditary disorder associated with severe lung infections. The median life expectancy of these patients is currently more than 35 years. In CF patients, platelets seem hyperactive. In our study we demonstrated they are not hyperactive, but they are less sensitive to inhibition. We could show this is probably a consequence of decreased levels of omega 3 fatty acids (docosahexaenoic acid). Why this omega 3 fatty acid is decreased remains to be shown. However, the CF lung plays an important role, as owe observed that mega 3 fatty acid levels normalize after lung transplantation.

In a second part of this work we studied liver disease in patients with CF. This liver disease in present in 20-30% of the patients and is the second cause of death. Currently, there are no good diagnostic tools to detect it. We demonstrated that the fibroscan (a new technique that measures the liver stiffness) can be used to detect liver disease in CF patients.

Why liver disease develops in CF is not clear. We documented that in CF, liver cirrhosis is a late event. Often there are earlier symptoms, like esophageal varices, that can lead to life-threatening hemorrhages. We have shown that these complications are the consequence of abnormalities in the liver's blood vessels: they are often too small or even absent.

Samenvatting

Bij patiënten met een leverziekte kunnen er stoornissen in de functie van de bloedplaatjes optreden. Zo keken we in een rattenmodel voor leverziekte met verminderde galafvloei (in galgang-geligeerde ratten) na wat het effect op die bloedplaatjes juist is. We vonden dat de bloedplaatjes eigenlijk normaal functioneren, maar dat ze worden tegengewerkt door iets in het plasma. We konden aantonen dat dit enzymen zijn die ADP (een component die bloedplaatjes normaal gesproken stimuleert) afbreken.

Mucoviscidose is een erfelijke aandoening die gepaard gaat met ernstige longinfecties. De levensverwachting van deze patiënten is momenteel meer dan 35 jaar. Bij patiënten met mucoviscidose lijkt het alsof de bloedplaatjes te actief zijn. Bij een studie van de bloedplaatjes van deze patiënten konden we aantonen dat ze niet per se hyperactief zijn, maar dat ze minder gevoelig zijn aan afremming van hun actie. We konden aantonen dat dit waarschijnlijk het gevolg was van een verlaging van een bepaald omega-3 vetzuur (docosahexaeen zuur). Waarom dit omega-3 vetzuur verlaagd is, moet nog worden uitgezocht. De long in mucoviscidose speelt hierbij mogelijk een belangrijke rol, aangezien we ook konden aantonen dat de omega-3 vetzuren normaliseren na een longtransplantatie.

In een tweede deel van dit doctoraat bestudeerden we

leverziekte bij patiënten met mucoviscidose. Dit komt voor bij 20-30% van de patiënten en is de tweede doodsoorzaak. Momenteel zijn er nog geen goede middelen om deze leverziekte op te sporen. Wij stelden vast dat het gebruik van de fibroscan (een nieuwe techniek waarbij de leverstijfheid wordt gemeten) hierbij een hulp kan zijn.

Waarom er juist leverziekte ontstaat bij mucoviscidose is niet geheel duidelijk. Men gaat er vanuit dat de gal te taai is, waardoor hij niet goed afvloeit uit de lever en er leverschade ontstaat. Er ontstaat dan leververlittekening (cirrose). Wij stelden nochtans vast dat bij patiënten met mucoviscidose en leverziekte deze leververlittekening slechts zeer laattijdig optreedt. Er zijn vaak al vroeger ernstige symptomen, zoals spataders in de slokdarm, die aanleiding kunnen geven tot levensbedreigende bloedingen. We toonden voor het eerst aan dat deze verwikkelingen het gevolg zijn van schade aan de kleine bloedvaten van de lever: deze zijn vaak te klein of zelfs afwezig.

Curriculum Vitae

Peter Witters was born in Schoten on July 16th 1981 and married to Annemie Monsieurs in 2009.

Studies

From October 2006- Present: PhD training Biomedical Sciences, Katholieke Universiteit Leuven

From August 2006- Present: Clinical trainee in the department of Paediatrics, University hospitals Leuven

From October 1999 to june 2006: Medical school Katholieke Universiteit Leuven, graduated summa cum laude

From 1993 until 1999, secondary school at the Sint-Michielscollege in Brasschaat

Research activities

October 2008-2010 FWO Flanders aspirant-investigator scholarship renewal.

October 2006-2008 recipient of FWO Flanders aspirant-investigator scholarship in preparation for a PhD thesis at the laboratory of Hepatology, Katholieke Universiteit Leuven, Belgium;

2001- 2003 student-investigator in the laboratory of experimental immunology, University of Leuven.

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